

NON-B, NON-C, NON-G HEPATITIS VIRUS GENE, POLYNUCLEOTIDE,  
POLYPEPTIDE, VIRUS PARTICLE, METHOD FOR ISOLATING VIRUS  
PARTICLE, AND METHOD FOR DETECTING VIRUS

Technical Field

The present invention relates to a gene, a polynucleotide, a polypeptide, an antibody, and an antigen, which have become available by discovery of an etiologic virus of blood-borne infectious hepatitis whose etiology could not be identified by conventional diagnostic methods, as well as methods for production, detection and assay of a virus gene, an antibody and an antigen, by utilizing the gene, the polynucleotide, the polypeptide, the antibody and the antigen which have been available, a virus particle and method for isolation of the virus particle.

Background Art

Hepatitis B virus (abbreviated as "HBV" hereinafter) and hepatitis C virus (abbreviated as "HCV" hereinafter) have been discovered so far as etiologic viruses of blood-borne infectious hepatitis. Diagnostic methods for the both viruses have already been established, and they were early introduced into the screening of blood for blood transfusion in this country. As a result, it has become possible to substantially completely prevent novel infection cases in blood recipients for the both hepatitis viruses.

However, even after the diagnostic methods for HBV and HCV were established, cases suspected of cryptogenic viral hepatitis constitute 5-10% of the whole hepatitis cases. These cases have not been considered to be caused by hepatitis A virus, which is a hepatitis virus of non-blood-borne infectious type, hepatitis E virus, hepatitis F virus, which has been reported only in India and its existence itself is doubted, and hepatitis D virus, which is a defective hepatitis virus, and possible existence of unknown hepatitis virus has been suggested.

Gene sequences of viruses considered to be etiologic agents of these hepatitis cases were reported in succession by Abbott, U.S.A. in 1995, and then by Genelabs Technologies in 1996, and designated as GBV-C and HGV, respectively. However, these viruses became to be considered identical one afterwards based on comparison of their sequences (abbreviated as "GBV-C/HGV" hereinafter). Studies about involvement of GBV-C/HGV in cryptogenic viral hepatitis are being actively performed also in this country.

As a result, it has thus far considered at least that GBV-C/HGV does not account for all of the causes of non-B, non-C hepatitis of unknown etiology, because expression of hepatitis symptoms has been slight in its infection cases while it may be transmitted via hematic route, and therefore an unknown virus might be responsible for the cryptogenic hepatitis.

Existence of an unknown hepatitis virus causing blood-borne infectious hepatitis has been suggested as described above, and it has been desired to discover this unknown hepatitis virus, and elucidate genetic, molecular-biological and epidemiological characteristics of the virus, thereby realizing more complete prophylactics, diagnostic methods and therapies for hepatitis.

In other words, in order to develop diagnostic methods and therapeutic methods of hepatitis caused by an unidentified virus, desired are to obtain genetic information of the virus, to determine virus-specific gene sequences and amino acid sequences, to determine locations of epitopes, to establish production methods of biological materials containing the epitopes, to establish production methods of antigens which specifically react with antiviral antibodies, to provide specific antibodies for the virus, to establish methods for isolating and collecting virus particles, to establish methods for treating the virus particles to attenuate their biological activity while maintaining their immunological activity, and to develop methods for assaying genes, antibodies, and antigens, and methods for producing neutralizing antibody-derived vaccines, which utilize the aforementioned biological materials derived from the virus to be obtained.

#### Disclosure of the Invention

An object of the present invention is to newly establish a diagnostic method and a treatment method of unidentified viral hepatitis whose etiologic virus has not been identified so far and therefore for which means for diagnosis, prevention and treatment have not been developed.

Another object of the present invention is to isolate an unidentified novel hepatitis virus gene to determine its gene sequence, thereby providing genes, polynucleotides, polypeptides, methods for isolating virus particles, virus particles, viral antigens, and antiviral antibodies, which can be utilized for diagnosis and treatment, and methods for detecting the virus.

The present inventors assumed that, in blood of patients suffering hepatitis of which cause cannot be identified by conventional diagnostic

methods of viral hepatitis, unknown hepatitis virus particles or a part thereof should be present. Based on this assumption, the present inventors attempted to isolate a viral gene from such blood. Specifically, the present inventors established a hypothesis that the viral gene would exist in blood of hepatitis patients, but does not exist in human genome and does not exist in blood of most of normal persons, or it does not exist before crisis of hepatitis, but exist after the crisis, searched for candidate genes in blood of hepatitis patients based on the hypothesis, and isolated them. Further, the candidate genes were examined for the following criteria, and a gene satisfying all of the criteria was ultimately considered as a novel viral genes:

- (1) the gene exists in blood of a plurality of patients of hepatitis of which cause has not been elucidated,
- (2) there are cases that blood transfusion recipients have become positive for the gene due to transfusion with blood positive for the gene while the gene has not existed in their blood before the blood transfusion, and crisis of hepatitis has been observed in them after the blood transfusion,
- (3) the obtained gene sequence is not homologous to gene sequences of known hepatitis viruses or other known viruses, and
- (4) when blood positive for the gene is analyzed by a density gradient centrifugation technique, which is widely used as a method for isolating and collecting virus particles, a fraction positive for the gene is found in a usual virus particle fraction.

Asymptomatic carriers, i.e., infection cases which are positive for a hepatitis virus but do not exhibit symptoms of hepatitis and appear healthy, are known for infection cases of known hepatitis viruses, and it was considered that such cases would be well possible for the infection cases of the virus of interest. Therefore, the criteria do not contain a criterion that "the genes do not found in normal persons."

According to the aforementioned criteria, the gene of the present invention was isolated, a sequence of the isolated gene was determined, and oligonucleotide sequences which could be utilized as PCR primers were searched.

By utilizing the obtained primers, a method for detecting the gene was established. Further, open reading frames existing in the gene sequence was identified, and amino acid sequences were specified. Density was also determined by density gradient centrifugation, and a method for collecting the virus was established. Thus, the present invention has been

completed.

That is, the present invention provides non-B, non-C, non-G hepatitis virus gene having a nucleotide sequence from which a sequence having a length of from about 3500 nucleotides to about 4000 nucleotides can be amplified by PCR utilizing an oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 57 and an oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 60 as primers, or PCR utilizing an oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 57 and an oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 61 as primers (also referred to as the gene of the present invention hereinafter).

The gene of the present invention preferably has a nucleotide sequence from which a sequence having a length of from about 3600 nucleotides to 3900 nucleotides can be amplified by the PCR.

Preferably, for the gene of the present invention, nucleotide sequences at 5' end and 3' end of the fragments amplified by the PCR respectively have 70% or more of homology to the nucleotide sequences of nucleotide number 3-300 and the nucleotide sequence of nucleotide number 2402-3739 of the nucleotide sequence shown in SEQ ID NO: 1. More preferably, the homology is 80% or more.

The present invention further provides non-B, non-C, non-G hepatitis virus gene of the following (a) or (b):

(a) a non-B, non-C, non-G hepatitis virus gene having a nucleotide sequence from which a sequence having a length of from about 200 nucleotides to about 350 nucleotides can be amplified by PCR utilizing an oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 6 and an oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 8 as primers, or

(b) a non-B, non-C, non-G hepatitis virus gene having a nucleotide sequence from which a sequence having a length of from about 200 nucleotides to about 350 nucleotides can be amplified by PCR utilizing an oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 7 and an oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 8 as primers.

The gene of the present invention is preferably a non-B, non-C, non-G hepatitis virus gene having the nucleotide sequence shown in SEQ ID NO: 1 or an allogeneic variant gene thereof. Examples of the gene include, for example, a non-B, non-C, non-G hepatitis virus gene having the nucleotide

sequence shown in SEQ ID NO: 45 (Genotype 1), a non-B, non-C, non-G hepatitis virus gene having the nucleotide sequence shown in SEQ ID NO: 46 (Genotype 2), a non-B, non-C, non-G hepatitis virus gene having the nucleotide sequence shown in SEQ ID NO: 47 (Genotype 3), a non-B, non-C, non-G hepatitis virus gene having the nucleotide sequence shown in SEQ ID NO: 48 (Genotype 4), a non-B, non-C, non-G hepatitis virus gene having the nucleotide sequence shown in SEQ ID NO: 49 (Genotype 5), a non-B, non-C, non-G hepatitis virus gene having the nucleotide sequence shown in SEQ ID NO: 50 (Genotype 6), a non-B, non-C, non-G hepatitis virus gene having the nucleotide sequence shown in SEQ ID NO: 51 (Genotype 7), a non-B, non-C, non-G hepatitis virus gene having the nucleotide sequence shown in SEQ ID NO: 52 (Genotype 8), a non-B, non-C, non-G hepatitis virus gene having the nucleotide sequence shown in SEQ ID NO: 53 (Genotype 9), and a non-B, non-C, non-G hepatitis virus gene having the nucleotide sequence shown in SEQ ID NO: 54 (genotype 10).

The present invention also provides a polynucleotide having a nucleotide sequence complementary to a nucleotide sequence of the gene of the present invention.

The present invention also provides an oligonucleotide comprising a nucleotide sequence recognized in the genes of the present invention and specific for the genes or a nucleotide sequence complementary thereto (also referred to the oligonucleotide of the present invention hereinafter). Specific examples of the oligonucleotide include, for example, a polynucleotide having the nucleotide sequence shown in SEQ ID NO: 2 (RD037), a polynucleotide having the nucleotide sequence shown in SEQ ID NO: 3 (RD038), a polynucleotide having the nucleotide sequence shown in SEQ ID NO: 4 (RD051), a polynucleotide having the nucleotide sequence shown in SEQ ID NO: 5 (RD052), a polynucleotide having the nucleotide sequence shown in SEQ ID NO: 6 (NG059), a polynucleotide having the nucleotide sequence shown in SEQ ID NO: 7 (NG061), and a polynucleotide having the nucleotide sequence shown in SEQ ID NO: 8 (NG063).

The present invention also provides a method for detecting a non-B, non-C, non-G hepatitis virus gene wherein PCR is performed by using the oligonucleotides of the present invention as primers.

Specific examples of the detection method include, for example, a method for detecting a non-B, non-C, non-G hepatitis virus gene wherein PCR is performed by using an oligonucleotide having the nucleotide

sequence shown in SEQ ID NO: 2 and an oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 3, or an oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 4 and an oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 5 as primers (first detection method), and a method for detecting a non-B, non-C, non-G hepatitis virus gene wherein PCR is performed by using an oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 6 and an oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 8, or an oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 7 and the oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 8 as primers (second detection method).

The present invention also provides a method for differentiating non-B, non-C, non-G hepatitis virus genotypes wherein both of the aforementioned first detection method and the second detection method are performed for one sample, and results obtained from the both gene detection methods are compared; and a method for differentiating non-B, non-C, non-G hepatitis virus genotypes wherein hybridization is performed by using an oligonucleotide having a sequence present in any one of Genotypes 1-6 and specific for any one of the genotypes.

The present invention further provides a polypeptide having an amino acid sequence encoded within an open reading frame present in a nucleotide sequence of the gene of the present invention (also referred to as the polypeptide of the present invention hereinafter).

Specific examples of the polypeptide of the present invention include, for example, a polypeptide having the amino acid sequence shown in SEQ ID NO: 9 and polypeptide having the amino acid sequence shown in SEQ ID NO: 10. The polypeptide of the present invention includes a polypeptide comprising an amino acid sequence which is found in the amino acid sequence shown in SEQ ID NO: 9 or 10, and specific for a non-B, non-C, non-G hepatitis virus. The polypeptide of the present invention preferably contains a non-B, non-C, non-G hepatitis virus-specific epitope.

Furthermore, the present invention also provides a method for isolating non-B, non-C, non-G hepatitis virus particles wherein the virus particles are isolated based on density of the non-B, non-C, non-G hepatitis virus particles; virus particles isolated by the method; and a non-B, non-C, non-G hepatitis virus peptides obtained from the virus particles.

In addition, the present invention further provides a recombinant

gene expression vector which comprises all or a part of a nucleotide sequence encoding the amino acid sequence shown in SEQ ID NO: 9 or 10; a transformant cell containing all or a part of a nucleotide sequence encoding the amino acid sequence shown in SEQ ID NO: 9 or 10; a non-B, non-C, non-G hepatitis virus antigen peptide or a fragment thereof expressed by the aforementioned transformant cell; and a method for producing a non-B, non-C, non-G hepatitis virus antigen peptide which comprises culturing the transformant cell under a condition that the non-B, non-C, non-G hepatitis virus antigen peptide is expressed, and collecting the expressed peptide.

Furthermore, the present invention provides a method for immunologically detecting a non-B, non-C, non-G hepatitis virus antibody using the polypeptide of the present invention (including that obtainable from virus particles) or the aforementioned virus particles as an antigen; a method for producing an antibody against the non-B, non-C, non-G hepatitis virus, which comprises immunizing an animal with the polypeptide of the present invention (including that obtainable from virus particles) or the aforementioned virus particles as an immunogen; antibodies obtained by the aforementioned method; and a method for immunologically detecting a non-B, non-C, non-G hepatitis virus antigen by using the aforementioned antibody.

The present invention further provides a vaccine containing a polypeptide having an amino acid sequence contained in an amino acid sequence encoded by an opening reading frame present in the nucleotide sequence shown in SEQ ID NO: 1, and containing an epitope sequence of a non-B, non-C, non-G hepatitis virus neutralizing antibody; and a vaccine containing the aforementioned virus particles.

The gene of the present invention is preferably also a non-B, non-C, non-G hepatitis virus gene having the nucleotide sequence shown in SEQ ID NO: 62 or an allogeneic variant gene thereof.

Also for this gene of the present invention, there are provided a polynucleotide having a nucleotide sequence complementary to a nucleotide sequence of the gene of the present invention; an oligonucleotide comprising a nucleotide sequence recognized in the gene of the present invention and specific for the gene or a nucleotide sequence complementary thereto; a method for detecting a non-B, non-C, non-G hepatitis virus gene wherein PCR is performed by using the aforementioned oligonucleotides as primers; a polypeptide having an amino acid sequence encoded within an open reading

frame present in a nucleotide sequence of the gene of the present invention; a recombinant gene expression vector; a transformant cell; a non-B, non-C, non-G hepatitis virus antigen peptide or a fragment thereof expressed by the aforementioned transformant cell; a method for producing a non-B, non-C, non-G hepatitis virus antigen peptide; a methods for immunologically detecting non-B, non-C, non-G hepatitis virus antibodies; a method for producing a non-B, non-C, non-G hepatitis virus antibody; an antibody; a method for immunologically detecting a non-B, non-C, non-G hepatitis virus antigen; and a vaccine.

The present invention will be explained in detail hereinafter.

The present invention relates to a so far unknown virus different from any of already known viruses. The term "non-B, non-C, non-G hepatitis virus" used for the present invention means an etiologic virus transmitted via blood-borne infection, and causing hepatitis symptoms, and it is a so far unknown virus different from hepatitis B, C, and G viruses, which are similarly blood-borne infectious viruses. Further, it is also different from hepatitis A virus, hepatitis E virus, and hepatitis F virus, which are of non-blood-borne infectious type, and hepatitis D virus, which is a defective virus, and hence it eventually has the same meaning as non-A, non-B, non-C, non-D, non-E, non-F, non-G hepatitis virus.

Hepatitis symptoms means symptoms generally representing hepatitis such as abnormal hepatic function values and appearance of icterus.

In the present invention, the virus of the present invention is defined as a hepatitis virus based on the context of the discovery of the virus of the present invention, but it does not necessarily means that major diseases caused by the virus of the present invention should be hepatitis.

The present inventors designated a non-B, non-C, non-G hepatitis virus having a non-B, non-C, non-G hepatitis virus gene having the nucleotide sequence shown in SEQ ID NO: 1 or a gene considered as an allogeneic variant thereof as "HNT22" virus (abbreviated as "HNT22" hereinafter), which is included in the gene of the present invention. HNT22 can be also defined as a non-B, non-C, non-G hepatitis virus having a non-B, non-C, non-G hepatitis virus gene of the following (a) or (b):

(a) a non-B, non-C, non-G hepatitis virus gene having a nucleotide sequence from which a sequence having a length of from about 200 nucleotides to about 350 nucleotides can be amplified by PCR utilizing an oligonucleotide



having a sequence shown in SEQ ID NO: 6 and an oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 8 as primers, or

(b) a non-B, non-C, non-G hepatitis virus gene having a nucleotide sequence from which a sequence having a length of from about 200 nucleotides to about 350 nucleotides can be amplified by PCR utilizing an oligonucleotide having a sequence shown in SEQ ID NO: 7 and an oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 8 as primers.

Further, a non-B, non-C, non-G hepatitis virus having a gene not included in the aforementioned gene, i.e., having the nucleotide sequence shown in SEQ ID NO: 62 or a gene considered as an allogeneic variant thereof is designated as "TUS01 virus" (abbreviated as "TUS01" hereinafter).

That is, HNT22 and TUS01 are so far unknown viruses isolated from non-A, non-B, non-C, non-D, non-E, non-F, non-G hepatitis patients, and they were separated from patients of hepatitis whose etiology has so far been considered unknown, and virologically or molecular biologically different from any known viruses.

HNT22 and TUS01 can be generically defined as a virus having a gene comprising a nucleotide sequence of the nucleotide number 3-300 of the nucleotide sequence shown in SEQ ID NO: 1 or a nucleotide sequence having high homology thereto in its 5' end portion, and a nucleotide sequence of the nucleotide number 2902-3738 of the nucleotide sequence shown in SEQ ID NO: 1 or a nucleotide sequence having high homology thereto in its 3' end portion, and having a length including the 5' end nucleotide sequence and the 3' end nucleotide sequence of about 3500 nucleotides to about 4000 nucleotides, preferably about 3600 nucleotides to about 3900 nucleotides.

In the present invention, hepatitis that is positive for HNT22 is called "HNT22 type hepatitis", and hepatitis that is positive for TUS01 is called "TUS01 type hepatitis".

It has been known that viruses are in general more likely to undergo mutations, and such mutations are more likely to be fixed in their genes compared with other higher organisms. Accordingly, a number of strains (allogeneic variants) may exist within the same species, and genotypes sharing a substantial gene sequence are also present. The terms HNT22 and TUS01 are used in the present invention as generic names of viruses including a possible great number of such strains and genotypes.

Because it has been known that viruses show high frequencies of mutations and fixation of such mutations in genes as described above,

definition of a virus by its gene sequences or amino acid sequences should be construed to include not only a virus containing specifically exemplified sequences but also those having gene sequences or amino acid sequences in a range considered to be substantially homologous. The substantially homologous range can be defined based on a ratio of homology which enables clear differentiation of one virus from the other virus by comparing their gene sequences or amino acid sequences. The substantially homologous range can also be defined by referring sequence diversity within a species of known similar viruses.

According to the aforementioned criteria, a gene having a nucleotide sequence having a homology to those of the HNT22 genes or the TUS01 genes of 55% or more, preferably 60% or more, more preferably 70% or more, particularly preferably 80% or more may be considered to be substantially homologous to the gene of the present invention, and fall within the scope of the present invention.

When genetic conservation within the HNT22 genes and homology with other viruses were actually determined for the HNT22 genes of the present invention, the homology within the HNT22 genes (conservation) was 55% or more, and the highest homology with other viruses was less than 60%.

Homology herein used means homology of nucleotide sequences and amino acid sequences, and it specifically means a ratio of a number of conformed nucleotides or amino acids to a number of nucleotides or amino acids contained in the whole sequences expressed in terms of percentage when sequences to be compared are aligned so that they should show the best conformance with a necessary deletion.

The hepatitis C virus (HCV), which causes blood-borne infectious hepatitis like the virus of the present invention, exhibited a homology of 60% or more within the species. Further, it has been reported that HCV of the same genotype exhibited a homology of 80% or more except for the partial high mutation region. From these facts, it is considered to be appropriate that the range of the gene of the present invention should be defined in terms of the aforementioned homology.

Similarly, as for the homology of the encoded amino acid sequence, those having a homology to the viruses of the present invention of usually 65% or more, preferably 70% or more, more preferably 80% or more, particularly preferably 90% or more are substantially homologous to the

viruses of the present invention, and included in them.

The genotypes existing in the HNT22 genes may variously classified, but, when based on the positions 1939-2160 in SEQ ID NO: 1 (referred to as nt1939-2160 hereinafter), an HNT22 gene having the nucleotide sequence shown in SEQ ID NO: 45 (Genotype 1), an HNT22 gene having the nucleotide sequence shown in SEQ ID NO: 46 (Genotype 2), an HNT22 gene having the nucleotide sequence shown in SEQ ID NO: 47 (Genotype 3), an HNT22 gene having the nucleotide sequence shown in SEQ ID NO: 48 (Genotype 4), an HNT22 gene having the nucleotide sequence shown in SEQ ID NO: 49 (Genotype 5), an HNT22 gene having the nucleotide sequence shown in SEQ ID NO: 50 (Genotype 6), and an HNT22 gene having the nucleotide sequence shown in SEQ ID NO: 51 (Genotype 7) can be mentioned.

By utilizing these nucleotide sequences, HNT22 viruses of a specific genotype can be detected or distinguished. As methods for differentiation, not only a method utilizing amplification by such a method as mentioned in Examples hereinafter and sequencing, but also a method by using an oligonucleotide having a sequence specific for any of the genotypes as a primer to amplify only a gene sequence of the corresponding genotype, a method by using such an oligonucleotide as probe to selectively detect the corresponding gene sequence, and a combination of these methods can be mentioned. Oligonucleotides, reaction conditions and the like that can be used for these methods can be selected according to methods known to those skilled in the art. For example, partial sequences characteristic of the genotypes (referred to as genotype-specific sequences hereinafter) can easily be selected by comparing the genotype sequences with one another. It is easy for those skilled in the art to select an oligonucleotide having a genotype-specific sequence or a sequence complementary thereto, and not hybridizing other HNT22 genotypes, or select a condition that an oligonucleotide having a genotype-specific sequence substantially cannot hybridize with other genotype sequences (Protein, Nucleic acid, Enzyme, Front Line of PCR, 1996, Kyoritsu Shuppan). Oligonucleotides selected as described above can be utilized as a primer or a probe to perform HNT22 genotype-specific amplification or detection.

The aforementioned HNT22 genes can be detected as one having a nucleotide sequence which can be amplified by PCR utilizing an oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 6

(NG059) and an oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 8 (NG063) as primers, and/or PCR utilizing an oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 7 (NG061) and an oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 8 (NG063) as primers.

In particular, the HNT22 genes having the nucleotide sequence shown in SEQ ID NO: 1 can be detected as one having a nucleotide sequence which can be amplified by PCR utilizing an oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 2 (RD037) and an oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 3 (RD038) as primers, and/or PCR utilizing an oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 4 (RD051) and an oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 5 (RD052) as primers.

The HNT22 genes of the present invention were obtained by the present inventors according to the following procedure.

It has been recognized that there are cases clearly exhibiting hepatitis symptoms while they are negative for any of known hepatitis virus markers. Such cases might be infection cases of unknown and non-elucidated hepatitis virus.

On the other hand, it may be thought that viruses are not present in healthy persons in principle. However, among hepatitis virus-infected patients, there may be asymptomatic carriers not exhibiting abnormality in their hepatic function and apparently healthy.

Therefore, the present inventors performed screening of genes present in hepatitis cases of which cause was indistinct, but absent in healthy persons or patients before crisis of hepatitis in order to search for unknown hepatitis virus. As a method for detecting genes present in one of groups to be compared as mentioned above, the known Representational Difference Analysis (Science 259, 946-950, 1993, abbreviated as "RDA method" hereinafter) was employed.

By determining sequences of the candidate genes obtained by the RDA method, the present inventors confirmed that the sequences were not homologous to sequences of known viruses.

Subsequently, a gene detection system which utilized an oligonucleotide primer constituting a part of the gene sequences was constructed, and the genes were confirmed to be detected in other hepatitis cases of which cause was not elucidated.

Further, a large number of sequences from cases positive for the genes were analyzed and compared to show that genotypes were present in the viruses, and a method for differentiating the genotypes utilizing sequences specific for the genotypes was constructed.

Furthermore, it was confirmed that the genes were not found in most of healthy persons. It was also confirmed that among the cases positive for the genes there were some cases which were negative for the genes before blood transfusion, but infected due to blood transfusion with blood positive for the genes and maintained as positive thereafter.

It was also verified that the genes were not those derived from the hosts.

From analyses utilizing density gradient centrifugation, a fraction positive for the genes was localized at a specific density as observed in other virus particles.

It was also verified that the virus was a single-stranded DNA virus by analysis based on the presence or absence of a reverse transcriptase reaction step, analysis utilizing a deoxyribonuclease, and experiments with restriction endonucleases.

Analyzing all of the above results, it was confirmed that the genes were transmitted by blood transfusion and maintained, hepatitis symptoms were observed in infection cases, positive persons were not so many among healthy persons, and sequences of the genes were not homologous to already known virus gene sequences. Taken together, the present inventors concluded that the viruses contained so far unknown hepatitis virus genes.

It was assumed that the aforementioned genes were a part of a virus gene based on their structures. The present inventors obtained the gene of the present invention by the known gene walking method based on the aforementioned gene sequences.

Embodiments of the present invention will be explained hereinafter by referring to the HNT22 genes, but it will be readily understood that the TUS01 genes can be used similarly.

The present invention provides an oligonucleotide or polynucleotide which has a sequence specific for the HNT22 genes, and can complementarily hybridize to the HNT22 genes under stringent conditions. Because of the characteristics of the oligonucleotide/polynucleotide, it can be effectively used as a primer for amplification of the HNT22 genes or as a probe for capturing or detecting the HNT22 genes in samples.

The term "specific sequence" herein used means a characteristic sequence portion which is present in a nucleotide sequence of a gene of interest or an amino acid sequence deduced from the nucleotide sequence, and distinguishable from sequences other than a sequence of interest. Whether a sequence is the characteristic sequence or not can be determined based on homology of sequence. Specifically, it may mean a sequence exhibits a homology of 10% or less with respect to known sequences other than the gene of interest having the same length, when the sequence is compared with the known sequences in a conventional manner, for example, by searching databases. Alternatively, it may be decided by determining if a gene of interest can be detected, identified and amplified by utilizing the sequence. Specifically, as for nucleotide sequences, when an oligonucleotide or polynucleotide having the sequence or an oligonucleotide or polynucleotide having a sequence complementary to the nucleotide sequence is used in a method for detection or amplification of genes known to those skilled in the art as a primer or probe, if the oligonucleotide or polynucleotide could detect, identify or amplify a gene of interest in a manner distinguishable from other genes with statistic significance, the sequence should be determined as specific for the gene of interest. As for amino acid sequences, whether a sequence is a specific sequence or not can similarly be determined based on homology of sequence. Specifically, a specific amino acid sequence is an amino acid sequence having such a length that it should show homology of about 10% or less when compared with known sequences. Alternatively, when an antibody raised by using a peptide composed of or containing an amino acid sequence encoded by a gene of interest as an antigen and verified to be bound to the amino acid sequence does not bound to or bound in a weaker degree with statistical significance to other antigens not derived from the amino acid sequence, the amino acid sequence can be determined as a sequence specific for the gene of interest.

The terms "polynucleotide" and "oligonucleotide" used for the present invention mean a nucleotide polymer having an arbitrary length, and include ribonucleotides and deoxyribonucleotides, for example, single- and double-stranded DNA, and single- and double-stranded RNA. These terms include not only unmodified polynucleotides, but also polynucleotides and oligonucleotides having a modification by, for example, methylation, capping, enzyme labeling, fluorescence labeling and the like.

The polynucleotides and the oligonucleotides of the present invention

include not only those derived from genomic DNA, but also polynucleotides and oligonucleotides obtained by synthesis, replication, transcription or amplification according to conventional methods.

The polynucleotides of the present invention include the exemplified sequences, sequences substantially homologous thereto, and polynucleotides having a sequence complementary thereto.

The oligonucleotides and the polynucleotides of the present invention can be used as primers for amplification of the HNT22 genes by PCR. In this method, a portion of the HNT22 genes between the primers can be amplified, thereby confirming that the portion of the HNT22 genes, i.e., HNT22 is present in a tested sample.

The present invention also provides an oligonucleotide probe or polynucleotide probe useful for capturing the HNT22 genes by hybridization, and detecting the HNT22 genes.

A suitable probe used for the capture or detection can be selected from the gene sequences in the same manner as the selection of the primers mentioned above, and a selected probe can be labeled in a conventional manner. It is also possible to detect a gene by capturing with the probe mentioned above a gene amplified with the primers mentioned above, and in particular, it becomes possible to distinguish HNT genotypes by utilizing a probe comprising a genotype-specific sequence.

The oligonucleotide of the present invention is usually an oligonucleotide having a relatively short sequence continuously present in a polynucleotide, and include those having the sequence in homologous or complementary polynucleotides. Its length is generally 6-50 nucleotides, preferably 10-30 nucleotides, more preferably 15-20 nucleotides.

Because the HNT22 genes show diversity as described above, a nucleotide sequence in a region where a nucleotide sequence is relatively well conserved among virus strains may be a nucleotide sequence specific for the genes. It is easy for those skilled in the art to select such a nucleotide sequence based on the nucleotide sequences disclosed herein. Specific examples of such an oligonucleotide include, for example, a polynucleotide having the nucleotide sequence shown in SEQ ID NO: 2 (RD037), polynucleotide having the nucleotide sequence shown in SEQ ID NO: 3 (RD038), polynucleotide having the nucleotide sequence shown in SEQ ID NO: 4 (RD051), polynucleotide having the nucleotide sequence shown in SEQ ID NO: 5 (RD052) polynucleotide having the nucleotide sequence shown in

SEQ ID NO: 6 (NG059), polynucleotide having the nucleotide sequence shown in SEQ ID NO: 7 (NG061), polynucleotide having the nucleotide sequence shown in SEQ ID NO: 8 (NG063) and the like.

The present invention also provides a method for detecting the HNT22 genes wherein PCR is performed by utilizing the oligonucleotides of the present invention as primers.

Specific examples of the methods include, for example, a method for detection of the HNT22 genes wherein PCR is performed by utilizing an oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 2 (RD037) and an oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 3 (RD038), or an oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 4 (RD051) and an oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 5 (RD052) as primers (first detection method), and a method for detection of the HNT22 genes wherein PCR is performed by utilizing an oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 6 (NG059) and an oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 8 (NG063), or an oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 7 (NG061) and the oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 8 (NG063) as primers (second detection method).

Samples used for PCR are prepared by extracting nucleic acids or DNA from biological samples such as plasma or sera collected from specimens by a known method. This preparation can be performed using a commercially available kit for extraction.

Conditions for PCR are suitably selected according to known PCR methods using a thermostable DNA polymerase.

Amplification products resulted from PCR can be detected by a known method such as electrophoresis, and the HNT22 genes can be detected based on the presence of the amplification products.

The present invention also provides a method for differentiating HNT22 genotypes wherein both of the aforementioned first detection method and the second detection method are performed for one sample, and results obtained from the both gene detection methods are compared, and a method for differentiating HNT22 genotypes wherein hybridization is performed by using an oligonucleotide having a sequence present in the genes of Genotypes 1-6 and specific for the corresponding genotype.

The sequence specific for each genotype can be selected by those



skilled in the art based on nucleotide sequences herein disclosed, or based on nucleotide sequences of genes detected by the methods for detection of genes mentioned above. Hybridization can be performed according to a known method. Specifically, nucleic acids are prepared from a biological sample, the prepared nucleic acids and an oligonucleotide are hybridized under stringent conditions, and the hybridized products containing the oligonucleotide are detected.

The specific oligonucleotides mentioned above are of course a part of examples, and other oligonucleotides which achieve the same object as mentioned above can be readily selected based on the HNT22 genes disclosed herein by utilizing techniques known to those skilled in the art. It is also easy to select an oligonucleotide which can be utilized for detecting both of the HNT22 genes and the TUS01 genes by combining the nucleotide sequences of the both genes disclosed herein and utilizing techniques known to those skilled in the art. For example, an oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 57 (NG054) and an oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 60 (NG065), or an oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 57 (NG054) and an oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 61 (NG021) can be mentioned as such oligonucleotides, and by performing PCR using these as primers, the HNT22 genes and the TUS01 genes can be detected. Similarly, those skilled in the art will be readily able to select oligonucleotides which can be utilized for detecting only the HNT22 genes, or only the TUS01 genes.

The present invention further provides a polypeptide having an amino acid sequence encoded within an open reading frame found in nucleotide sequences of the genes of the present invention (referred to as the polypeptide of the present invention hereinafter).

The term "polypeptide" herein means a linearly linked polymer of amino acids, and its length is not particularly limited. Accordingly, the polypeptide of the present invention includes any of those usually referred to as oligopeptides, proteins, and peptides, and as for their origin, it includes not only naturally occurring ones, but also fragments of the naturally occurring ones and those prepared by various means such as chemical syntheses and recombinant expression techniques. As described above, it has been known that viruses show differences of gene sequences even within the same species, and therefore similarly show differences in amino acid

sequences. Accordingly, homology of an amino acid sequence necessary for determining the sequence as one of the viruses of the present invention is 65% or more, preferably 70% or more, more preferably 80% or more, and particularly preferably 90% or more.

The term "open reading frame" (abbreviate as "ORF" hereinafter) used for the present invention means a region of nucleotide sequence encoding a polypeptide or a part thereof. This sequence is transcribed and translated into a polypeptide when it is placed under a suitable condition. Boundaries of a coding sequence are an initiation codon at the 5' end and a termination codon at the 3' end.

It is known in the art to deduce, from a gene sequence, an encoded amino acid sequence, and therefore what is important is to define ORF in a gene sequence. ORF can be determined as follows.

A triplet nucleotide sequence which could be an initiation codon, where translation into an amino acid starts, should be identified in an obtained gene sequence. This location is assumed as an initiation codon, and ORF encoding a polypeptide of a certain size is determined within a range where a termination codon does not appear. Examples of amino acid sequences which are obtained in this manner include the amino acid sequences shown in SEQ ID NOS: 9 and 10.

The polypeptide of the present invention preferably comprises an amino acid sequence specific for HNT22. An amino acid sequence specific for HNT22 can be selected by those skilled in the art based on amino acid sequences encoded by the nucleotide sequences disclosed herein, or amino acid sequences encoded by nucleotide sequences of genes detected by the aforementioned methods for detection of genes.

The polypeptide of the present invention more preferably contains an HNT22-specific epitope.

The term "epitope" used in the present invention means an antigenic determinant. The antigenic determinant is a site which is present in an antigen molecule and directly bound to an antibody, and it is constituted by at least three amino acids, usually 5-10 amino acids in the steric configuration. Methods for determining a location of epitope and its configuration in a steric structure of a peptide are known in the art, and can be performed by those skilled in the art. Accordingly, the term "HNT22-specific epitope" used in the present invention means an antigenic determinant specific for HNT22, and it is an epitope composed of an amino

acid sequence specific for HNT22. This epitope is usually composed of eight or more contiguous amino acids.

The expression "containing an epitope" used in the present invention means that such an epitope sequence as defined above is contained as a part of polypeptides, and specific examples of such polypeptides containing an epitope include those composed of an epitope sequence bound to a carrier peptide or a linker peptide.

The polypeptide of the present invention can be effectively used as an antigen for antibody tests or an immunogen for preparing antiviral antibodies. It is expected that viral polypeptides include an amino acid sequence which does not have an amino acid sequence specific for the virus and may cause non-specific antibody reactions when this portion is used as an antigen. Therefore, it is important also in the present invention to specify a sequence suitable for antibody tests from the whole amino acid sequence. As a method for identifying such a specific sequence, i.e., an epitope sequence, there is available a method wherein peptides each having a certain length that cover the whole sequence are synthesized, and presence or absence or degree of reaction of each peptide with an antiviral antibody, namely, a serum of a patient infected with the virus is utilized. Instead of the above method involving synthesis, a phage library using  $\lambda$ gt11 can be prepared and screened with a blood serum of the patient.

The present invention also provides a method for isolating non-B, non-C, non-G hepatitis virus particles wherein the particles are isolated based on density of the particles, virus particles isolated by the aforementioned method, and a non-B, non-C, non-G hepatitis virus peptide obtained from the aforementioned virus particles.

The term "virus particles" used in the present invention means a fraction positive for the gene of the present invention which is collected as a fraction of a particular density by density gradient centrifugation, which is used as a usual method for isolation of virus particles, and it is not limited to those having a particle structure in a morphological sense, or infectious particles.

Viruses have densities corresponding to their particle structure, and they can be separated from other coexisting substances based on the density. The most usual method for isolating virus particles based on density is a method utilizing density gradient centrifugation. In this method, a gradient density carrier layer is formed in a centrifugation tube using

sucrose or the like, then a fraction containing viruses is overlaid on the layer, and ultracentrifugation is performed. This technique is based on the fact that, when viruses migrated by centrifugal force reach a layer having the same density as the viruses, the centrifugal force and buoyancy reach equilibrium, and the viruses stop the migration, thereby the viruses are concentrated in that fraction.

The polypeptide can be obtained from virus particles obtained by the method mentioned above by a known method. For example, virus particles are treated with a denaturation agent, and a polypeptide is separated from other components.

The present invention also provides a gene expression vector into which the HNT22 gene or a partial sequence thereof is integrated. The term "recombinant expression vector" herein used means a vector for inserting an exogenous polynucleotide into a gene of a host cell so that the polynucleotide could be expressed. Specifically, it is a vector having control sequences enabling the integration of the polynucleotide. The term "partial" means that the sequence is composed of a part of the genes which encodes a peptide sufficient for exhibiting antigenicity.

The expression vector can be effectively used for obtaining a viral peptide having immunological activity or biological activity. Various vectors for integrating a gene desired to be expressed are currently known, and various host cells for the integration of the vectors and consequent expression of peptides are also known.

The gene expression vector provided by the present invention is a recombinant expression vector having an HNT22 genome or ORF thereof, wherein the ORF is operably linked to regulatory sequences compatible with a desired host. A recombinant gene expression system can be constructed by using the expression vector.

The term "transformant cell" herein used means a cell of which gene is integrated with all or a part of a gene of the present invention, and which can express all of or a part of a polypeptide encoded by the gene of the present invention.

The transformant cell provided by the present invention can be obtained by directly introducing a polynucleotide containing all or a part of the gene of the present invention, or introducing a recombinant expression vector integrated with such a polynucleotide into a host cell to transform the host cell. In the present invention, known transfer vectors and host cells

can be used.

The polypeptide produced by the transformant cell (HNT22 virus antigen peptide) can be obtained by culturing the transformant cell under a condition that the HNT22 virus antigen peptide can be expressed, and collecting the expressed polypeptide.

The present invention provides a method for immunologically detecting an HNT22 antibody by using HNT22 particles, an HNT22 polypeptide, an HNT22 epitope, or a polypeptide containing the HNT22 epitope as an antigen. As methods for detection of antibodies using peptide antigens, there can be mentioned immunonephelometry, enzyme immunoassay, radiometric immunoassay, agglutination method and the like, and these methods can be used for the present invention.

The present invention also provides a method for preparing an HNT22 antibody by utilizing HNT22 particles, an HNT22 polypeptide, an HNT22 epitope, or a polypeptide containing the HNT22 epitope, which is purified or partially purified, as an immunogen, and the HNT22 antibody. As a method for preparing the antibody by utilizing purified or partially purified virus particles or polypeptides, conventional methods can be used.

The present invention provides a method for immunoassay of an HNT22 antigen by utilizing an antibody specifically binding to HNT22 and a kit therefor. As an example of the method for assaying the antigen by using the antibody, there can be mentioned a method comprising:

- (1) a step of reacting an antigen in a sample with an antibody capable of binding to HNT22 which are immobilized on a solid phase of a reaction vessels or reaction carrier, so that the antigen in the sample should be captured by the antibody bound to the solid phase through antigen-antibody reaction,
- (2) a step of further reacting the captured antigen, after an appropriate washing step, with an appropriately labeled antibody capable of binding to HNT22 and having specificity for HNT22, and
- (3) a step of detecting the binding antibody, after an appropriate washing step, by utilizing the function of the labeled antibody.

As another method, there can be mentioned a method comprising:

- (1) a step of reacting a sample to be assayed with a suitably labeled polypeptide having an HNT22 epitope at a defined concentration for a certain period of time,
- (2) a step of reacting the sample which has undergone the step (1) with an

HNT22 antibody immobilized on a solid phase of a reaction vessel or reaction carrier, so that the antigen in the sample should sufficiently bind to the antibody on the solid phase, and

(3) a step of separating labels bound to the reaction vessel or reaction carrier and unbound labels, and measuring the bound labels or the unbound labels.

Other methods utilized for assaying the viral antigen such as agglutination method, reversed passive agglutination method, enzyme immunoassay, radiometric immunoassay, and fluorescence polarization method and the like can also be used for the present invention.

The present invention provides a vaccine comprising purified HNT22 particles, a polypeptide containing a neutralizing antibody epitope obtained from the purified virus particles, HNT22 particles and a polypeptide containing a neutralizing antibody epitope obtained by using recombinant expression, a purified polypeptide and a fragment containing a neutralizing antibody epitope obtained from the expressed virus particles or the expressed polypeptide, or a polypeptide containing a neutralizing antibody epitope chemically synthesized based on the gene sequence of the virus.

For the preparation of the vaccine of the present invention, a protein bearing an antigenically active region of HNT22 obtained by the aforementioned method for synthesis or recombinant expression based on the genes disclosed herein can be used. As for known viruses, peptide antigens and recombinant antigens containing epitopes of envelope antigens have such activity. Other structural protein antigens also may have such activity by themselves or when combined with other antigens. Specifically, peptide antigens or recombinant antigens containing epitopes of envelope antigens contain the epitope. Other structural protein antigens may also contain a neutralizing antibody epitope by themselves or when combined with other antigens.

By using an antigen which is expressed by utilizing the gene of the present invention or HNT22 particles purified by the isolation method of the present invention, a multivalent vaccine containing a plurality kinds of neutralizing antibody epitopes can be obtained. When isolated and purified particles are used, the virus should be inactivated, and it can be realized by a known method such as treatment with formalin.

The method for preparing a vaccine containing an immunogenic polypeptide as an active ingredient may also use a known one. That is, it is prepared as a liquid preparation, or a suspension as an injection solution, or

a solid preparation suitable for being dissolved or suspended in a liquid before injected. The immunologically active ingredient is mixed with a suitable excipient. As the excipient, water, physiological saline, dextrose, glycerol, ethanol and the like can be mentioned. An auxiliary in a small amount can also be added as required. As the auxiliary, humectants, emulsifiers, pH buffers, adjuvants and the like can be mentioned. Examples of the adjuvant include, for example, aluminum hydroxide, N-acetyl-muramyl-L-threonyl-D-isoglutamine, N-acetyl-muramyl-L-alanyl-D-isoglutamine and the like.

Administration formulas of these vaccines affording the desired effect can be suitably defined. Dose per administration is generally 5  $\mu$ g to 250  $\mu$ g, and it is decided depending on body weight and ability of immunological response of individuals to be administered, desired degree of antibody induction and the like. Frequency of administration may also be selected according to similar standards.

#### Brief Explanation of the Drawings

Figs. 1-5 show comparison of sequences of 396 nucleotides between NG001 (SEQ ID NO: 40) and a sequence complementary to RD052 (SEQ ID NO: 5) (corresponding to nt1862-2257 in SEQ ID NO: 1) of the genes obtained from 75 of HNT22 positive samples.

The whole sequence of the corresponding region (nt78-299) of #22 (SEQ ID NO: 11) is shown at the top of the sequences of the samples, and thereunder nucleotides of Samples 1-75 different from #22 are shown with alphabet while positions of the same nucleotide are shown with dots (·). Based on homology of sequences (corresponding to nt1939-2160 in SEQ ID NO: 1, nt78-299 in SEQ ID NO: 11), they can be classified into four groups, group of Samples 1-49 (designated as Genotype I), group of Samples 50-73 (designated as Genotype II), Sample 74 and Sample 75.

The locations of the primers used in the Examples 2 and 3 are shown in the top row. It can be seen that mismatches are commonly present at the 3' end side of the primers of RD037 (SEQ ID NO: 2) and RD051 (SEQ ID NO: 4) in Genotype II and Samples 74 and 75.

Fig. 6 represents extension of #22 clone sequence using gene walking (Example 4).

Fig. 7 represents comparison of:  
(1) HNT22 gene sequence in blood for transfusion (SEQ ID NO: 11),

(2) HNT22 gene sequence derived from a patient two weeks after blood transfusion, and

(3) HNT22 gene sequence derived from a patient four weeks after blood transfusion

for HNT22 infection cases caused by blood transfusion (Example 7). The compared sequences are completely consistent with one another.

Fig. 8 represents the results of searching of open reading frame (ORF) of the HNT22 genes (Example 10).

Upper 3 frames: candidate locations of initiation codon and termination codon in the nucleotide sequence shown in SEQ ID NO: 1. The short vertical bars indicate initiation codons, and the long vertical bars indicate termination codons. The frames show sequences each of which open reading frame is shifted by one nucleotide for every sequence. Long ORFs were found in the first and the second frames.

Lower 3 frames: candidate locations of initiation codon and termination codon in a sequence complementary to the nucleotide sequence shown in SEQ ID NO: 1. Long ORF was not recognized in any of the frames.

Fig. 9 represents hydrophilicity/hydrophobicity scores of polypeptides based on the amino acid sequences encoded by the open reading frames 1 and 2.

Fig. 10 represents a molecular phylogenetic tree based on the nucleotide sequences of the genes from HNT22 positive cases obtained in Example 12.

Figs. 11 and 12 represent comparison of nucleotide sequences of the genes from HNT22 positive cases obtained in Example 12.

Fig. 13 represents locational relation of the primers and the clones used for the sequencing of the full length TUS01 gene. Names of the clones whose sequences were determined are indicated in the boxes. Names of the primers used for the amplification are indicated on the left and right tops of the boxes, and nucleotide numbers when the first nucleotide of the 5' end is defined as nucleotide 1 are indicated in parentheses.

Fig. 14 represents the results of searching of open reading frame (ORF) of the TUS01 gene.

Fig. 15 represents comparison of 5' end region sequences of the HNT22 gene and the TUS01 gene.

Fig. 16 represents comparison of 3' end region sequences of the



HNT22 gene and the TUS01 gene.

#### Best Mode for Carrying Out the Invention

The present invention will be explained in detail hereinafter with reference to the following examples, but of course the present invention is not limited to these examples.

#### Example 1: Isolation of Clone #22

For 3 cases clinically confirmed as post-transfusion hepatitis of non-A, non-B, non-C, non-D, non-G (Cases 1-3), genes present only in blood samples of the patients after crisis of post-transfusion hepatitis by the RDA method (subtraction) using blood of the patients upon or after the crisis of post-transfusion hepatitis and blood of the same patients before blood transfusion or before the crisis as samples. The RDA method is a method for detecting genes present only one part of comparison groups as described above, and it is a method for efficiently searching genes different between a tester which is a sample, and a driver which is a control.

In this example, a sample after crisis of hepatitis of Case 2 among the above-mentioned three cases clinically diagnosed as post-transfusion hepatitis whose cause was indistinct (see Example 6 for details) were used as a tester (A), and a sample of the same case before hepatitis crisis after blood transfusion (B1) and a sample of other acute hepatitis B patients (B2) were used as drivers, and genes which had first appeared in the blood of the patient after crisis of post-transfusion hepatitis were discovered.

The procedure will be explained in detail hereinafter.

##### (1) Extraction of nucleic acids

The above post-transfusion hepatitis case (Case 2) was negative for any of known hepatitis virus markers. Its blood serum (mixture of 50  $\mu$ l each of blood sera eight weeks and ten weeks after the blood transfusion of Case 2) was used as an unknown hepatitis virus positive tester (A).

As controls negative for the unknown virus, blood serum of the same patient from who (A) was obtained but before the crisis of hepatitis (two weeks after the blood infusion, B1), and serum of another patient of acute hepatitis B (B2) were used as drivers. First, nucleic acids were extracted from 100  $\mu$ l each of the sera of the tester and drivers by using a commercially available kit for nucleic acid extraction (ISOGEN-LS, Nippon Gene). That is, each serum (100  $\mu$ l) and a nucleic acid extraction solution (300  $\mu$ l) were

taken into a 1.5-ml Eppendorf tube, stirred for mixing for one minute, and left at room temperature for 5 minutes. After the mixture remained on the wall of the tube was fallen by centrifugation, the mixture was added with chloroform (80  $\mu$ l), stirred for one minute, and left at room temperature for 5 minutes. After the reaction was completed, the mixture was subjected to centrifugation for 15 minutes at 12000 revolutions/minute to afford a supernatant. The supernatant (210  $\mu$ l) was taken in another Eppendorf tube, added with glycogen (20 mg/ml, 1  $\mu$ l, Boehringer Mannheim) and isopropanol (200  $\mu$ l, Wako Pure Chemicals Industries), and mixed. After the mixture was left at room temperature for 10 minutes for reaction, it was subjected to refrigerated centrifugation at 12000 revolutions/minute (4°C) for 10 minutes to afford precipitates, which was washed with 70% ethanol (Wako Pure Chemicals Industries) and dried in air.

## (2) Synthesis of cDNA

The nucleic acid obtained by the above procedure was dissolved in DEPC-water (10  $\mu$ l, deionized water treated with diethyl pyrocarbonate, Sigma). This solution was added with a randomized hexamer solution (50 ng/ $\mu$ l, 1  $\mu$ l), warmed at 70°C for 5 minutes, and then promptly ice cooled. After the cooling, a first strand buffer at 5-fold concentration (4  $\mu$ l), 0.1 M DTT (2  $\mu$ l), 10 mM dNTP (1  $\mu$ l), RNase inhibitor (1  $\mu$ l, 40 U/ $\mu$ l, RNasin, PROMEGA), and reverse transcriptase (1  $\mu$ l, 200 U/ $\mu$ l, SuperScript II, GIBCO-BRL) were added to the solution, and allowed to react at 37°C for 60 minutes for synthesis of cDNA of the first strand. Then, to a tube containing the cDNA, DEPC-water (91  $\mu$ l), a second strand buffer at 5-fold concentration (30  $\mu$ l), 10 mM dNTP (3  $\mu$ l), E. coli DNA ligase (1  $\mu$ l, 10 U/ $\mu$ l), E. coli DNA polymerase (4  $\mu$ l, 10U/ $\mu$ l), and E. coli RNaseH (1  $\mu$ l, 20 U/ $\mu$ l) were added and mixed, and allowed to react at 16°C for two hours. Then, the reaction mixture was added with T4 DNA polymerase (2  $\mu$ l, 5 U/ $\mu$ l), and allowed to react at 16°C for 5 minutes to synthesize second strand cDNA. The reaction was stopped by adding EDTA (5  $\mu$ l, 0.5 M). Subsequently, a mixture of phenol and chloroform (150  $\mu$ l, 1:1) was added to the mixture in the tube and mixed, and subjected to centrifugation at 15000 revolutions/minute at room temperature for 5 minutes to separate proteins as precipitates. The obtained supernatant (156  $\mu$ l) was added with glycogen (1  $\mu$ l, 20 mg/ml), 7.5 M ammonium acetate (78  $\mu$ l, Wako Pure Chemicals Industries), and cold ethanol (562  $\mu$ l, Wako Pure Chemicals Industries), and

immediately centrifuged at 15000 revolutions/minute at room temperature for 20 minutes to afford nucleic acids as precipitation fraction. The precipitates were washed with cold 70% ethanol (600  $\mu$ l), dried in air, and then dissolved in TE buffer (50  $\mu$ l, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA). The solution was then subjected to gel filtration using Microspin S-400 HR Column (Pharmacia). The obtained eluent (50  $\mu$ l) was added with 1/10 volume of 3M sodium acetate (pH 5.2) and 2.5 volumes of ethanol, left stand at -80°C for 20 minutes, and centrifuged at 15000 revolutions/minute at 4°C for 20 minutes to collect a cDNA/DNA fraction as precipitates.

### (3) *Sau*3AI digestion

The cDNA/DNA fraction collected by the above procedure was dissolved in water (45  $\mu$ l), added with H buffer at 10-fold concentration (5  $\mu$ l, buffer attached to the restriction endonuclease *Sau*3AI, Takara Shuzo), and *Sau*3AI (4  $\mu$ l, 12 U/ $\mu$ l, Takara Shuzo) and mixed, and allowed to react at 37°C for 1.5 hours. After the reaction was completed, the reaction mixture was added with a mixture of phenol and chloroform (40  $\mu$ l, 1:1), stirred, and subjected to centrifugation at 15000 revolutions/minute at room temperature for 15 minutes. The supernatant (60  $\mu$ l) was added with 1/10 volume of 3 M ammonium acetate, pH 5.2 and 2.5 volumes of ethanol, left stand at -80 °C for 20 minutes, and subjected to centrifugation at 15000 revolutions/minute at 4°C for 20 minutes to collect nucleic acids as precipitates.

### (4) Ligation of adapter

Adapters were introduced into the both ends of the fragments by ligating adapters R-Bam24 and R-Bam12 that were compatible with the nucleotide sequences of the ends cleaved with the restriction enzyme *Sau*3AI. That is, the nucleic acid fraction precipitates obtained by the above procedure was dissolved in water (16.1  $\mu$ l), and added with a T4 ligase buffer at 10-fold concentration (3  $\mu$ l, NEB), the adapter R-Bam24 (6.0  $\mu$ l, 10 OD/ml), and the adapter R-Bam12 (3.0  $\mu$ l, 10 OD/ml), then the ambient temperature was lowered from 50°C to 10°C over one hour, and the mixture was added with T4 ligase (1.5  $\mu$ l, 400 U/ $\mu$ l, NEB), and allowed to react at 16°C overnight.

### (5) PCR amplification

Then, the gene fragments were treated with DNA polymerase to

obtain gene fragments which were completely double-stranded over the full length, and these gene fragments were amplified by PCR using R-Bam24 as primer as follows. The nucleic acid fraction introduced with the adapters by the above procedure was warmed at 70°C for 15 minutes to inactivate the T4 ligase in the reaction mixture. Then, TaKaRa EX Taq Polymerase buffer at 10-fold concentration (20 µl, Takara Shuzo), 2.5 mM dNTP (24 µl), water (149.3 µl), and R-Bam24 (5.2 µl, 10 OD/ml) were added to the mixture, and allowed to react at 70°C for three minutes, and then TaKaRa EX Taq Polymerase (1 µl) was added to perform PCR with the following conditions. That is, after treatment at 72°C for 5 minutes, a cycle of 95°C for 1 minute and 72°C for 3 minutes was repeated for 30 cycles, followed by treatment at 72°C for 7 minutes and cooling to 4°C. After the completion of the reaction, nucleic acids were extracted with phenol and chloroform in the same manner as described above, and precipitated with ethanol. The precipitates of the PCR products obtained from eight tubes were combined and dissolved in TE buffer (100 µl), and subjected to gel filtration in the same manner as described above to afford an eluted solution, which was subjected to precipitation with ethanol.

#### (6) Removal of adapter

The PCR products precipitated with ethanol after the gel filtration were dissolved in water (90 µl). To this solution was added H buffer at 10-fold concentration (10 µl) and then *Sau3AI* (7 µl), and allowed to react at 37°C for 15 minutes. Then, the nucleic acids were collected as precipitates in the same manner as described above, then subjected to gel filtration using MicroSpin S-400 HR Column as described above, and precipitated with ethanol and collected. The product was dissolved again, and its absorbance at 260 nm was measured to confirm the yield.

#### (7) Ligation of adapter (J-Bam24/J-Bam12) to tester

Then, the adapters J-Bam24 and J-Bam12 which are compatible with *Sau3AI* sequence were connected only to the tester amplified gene fragments as follows. That is, among the amplification products whose R-Bam24 and R-Bam12 tester were removed, those derived from the tester were added with T4 ligase buffer at 10-fold concentration (3 µl per 1.0 µg of the PCR products), and further added with the adapters J-Bam24 (13.2 µl) and J-Bam12 (6.6 µl) and water (4.9 µl). The ambient temperature was

lowered from 50°C to 10°C over one hour to anneal them. To this reaction mixture, T4 DNA ligase (400 U/μl, NEB, 1.0 μl) was added, and allowed to react at 16°C overnight to ligate the adapters to the *Sau3AI* cleaved ends. Then, the mixture was treated at 70°C for ten minutes to inactivate the ligase.

(8) Hybridization of tester amplified gene fragments with adapter and driver amplified gene fragments without adapter

The amplification products derived from the tester to which adapters were connected and the amplification products derived from the drivers of which adapters were removed by the procedure described were denatured by heat, thereby completely made into single-stranded DNA, and then re-associated as follows. First, a mixture of phenol and chloroform (1:1, 30 μl) was added to the reaction mixture to remove proteins. To the obtained supernatant (17 μl), a driver gene amplification product whose adapters were removed (40 μg) was added, and precipitated with ethanol. The precipitates were added with EE buffer at 3-fold concentration (4 μl) and dissolved therein, and overlaid with mineral oil (30 μl, Sigma), and the DNA was denatured by a treatment at 98°C for ten minutes. After the denaturation, the mixture was immediately cooled with ice, and added with 5 M sodium chloride (Wako Pure Chemicals Industries, 1 μl). Then, hybridization was performed at 67°C for about 22 hours.

During the above procedure, for genes commonly present in the tester and the drivers, like genes derived from human, original combinations of DNA, i.e., double-stranded DNA composed of re-associated two DNA strands derived from the tester, or two DNA strands derived from the driver (homo-double-stranded DNA), and double-stranded DNA composed of one DNA strand derived from the tester and one DNA strand derived from the driver (hetero-double-stranded DNA) were formed. In contrast, for DNA present only in the tester (considered to be an exogenous gene), hetero-double-stranded DNA was not formed and only homo-double-stranded DNA derived from the tester was formed because the corresponding allogeneic gene was not present in the driver. Further, only the double-stranded DNA having a gene sequence derived from the tester had the adapter sequences in both of the strands.

### (9) Amplification

A sample undergone the hybridization in the above procedure was added with water (15  $\mu$ l), and then nucleic acids in the sample were precipitated with ethanol in the same manner as described above. The precipitates were added with 1/2 TE buffer (20  $\mu$ l, 5 mM Tris-HCl, pH 7.5, 0.5 mM EDTA), and dissolved therein. To this solution (2  $\mu$ l), Ex Taq Polymerase buffer at 10-fold concentration (20  $\mu$ l), 2.5 mM dNTP (240  $\mu$ l), water (147.7  $\mu$ l), and TaKaRa EX Taq polymerase (1  $\mu$ l) were added, and allowed to react at 72°C for 5 minutes. During this reaction, DNA was synthesized for the portions remained as a single strand in the double-stranded DNA formed in the above operation, and terminuses were blunt-ended. This sample was added with J-Bam-24 (5.3  $\mu$ l, 10 OD/ml) as a primer, and PCR was performed by 10 cycles of a cycle of 95°C for 1 minute and 70°C for 3 minutes, and leaving the mixture at 70°C for 7 minutes and then at 4°C. Nucleic acids were extracted from the amplified sample in the same manner as described above by using a mixture of phenol and chloroform, and precipitated with ethanol by using glycogen as a coprecipitating agent. Under this condition, only the genes derived from the tester to which the J-Bam24 was ligated as adapter can be amplified by utilizing the added J-Bam24 as a primer. As a result, DNA derived from the homo-double-stranded DNA of the tester specific genes can be amplified for the both DNA strands, and hence exponentially amplified. On the other hand, the hetero-double-stranded DNA constituted by the genes common to the tester and the driver is amplified just multiplicatively only for the DNA strand having the driver gene sequence of which template is the tester. The DNA strands derived from the homo-double-stranded DNA composed only of the driver genes cannot be amplified because the added primer cannot hybridize to them. Accordingly, the homo-double-stranded DNA having the tester specific gene sequences becomes predominant in the reaction mixture after the amplification.

### (10) Mung bean nuclease treatment

In order to obtain only tester specific genes except for single strand driver gene sequence DNA present in the reaction mixture (precisely, a few double-stranded DNA derived from the driver will remain), mung bean nuclease (abbreviate as "MBN" hereinafter) treatment was performed as follows. Nucleic acid fractions obtained by precipitating from hybridization

samples of the tester A and the driver B1, or the tester A and the driver B2 were each added with TE buffer of a 1/2 concentration (10  $\mu$ l) and dissolved therein. 5  $\mu$ l was taken from each solution, added with MBN buffer at 10-fold concentration (2  $\mu$ l), water (13  $\mu$ l), and MBN (0.5  $\mu$ l, 300 U/ $\mu$ l, Takara Shuzo), and allowed to react at 37°C for 30 minutes. After the completion of the reaction, 50 mM Tris-HCl, pH 8.9 (80  $\mu$ l) was added to the reaction mixtures and allowed to react at 95°C for 5 minutes to inactivate the nuclease. Aliquots of 5  $\mu$ l and 10  $\mu$ l taken from the above reaction mixtures, and the above hybridization sample not subjected to the nuclease treatment (1  $\mu$ l) were each added with Ex Taq Polymerase buffer at 10-fold concentration (20  $\mu$ l), 2.5 mM dNTP (24  $\mu$ l), the primer J-Bam24 and TaKaRa Ex Taq DNA Polymerase (1  $\mu$ l), and further added with water to a total volume of 200  $\mu$ l, and PCR was performed with the following condition. Namely, a reaction cycle of 95°C for 1 minute and 70°C for 3 minutes was repeated for 20 times, followed by treatment at 70°C for 7 minutes and cooling to 4°C. 10  $\mu$ l was taken from the PCR products, and subjected to gel electrophoresis (1  $\times$  TBE buffer) on 2.5% NuSieve 3:1 agarose (FMC BioProducts, USA). Since the results were the same for the combination of A and B1 and the combination of A and B2, only the combination of A and B1 will be described hereinafter.

Nucleic acids were extracted from the remained amplification products by the extraction method mentioned above using a mixture of phenol and chloroform, precipitated with ethanol, and collected. The precipitates were added with TE buffer (45  $\mu$ l) and dissolved therein. This solution was subjected to gel filtration using MicroSpin S-400 HR Column mentioned above, and the eluted solution was subjected to ethanol precipitation.

#### (11) Removal of adapter

The precipitates obtained above were dissolved in water (63  $\mu$ l), added with H buffer at 10-fold concentration (7  $\mu$ l) and *Sau3AI* (6  $\mu$ l, 12 U/ $\mu$ l), and allowed to react at 37°C for 1.5 hours to cleave the J-Bam24 and J-Bam12 adapter moieties. Proteins were removed from the reaction mixture by using a mixture of phenol and chloroform as described above, and nucleic acids were precipitated with ethanol. The precipitates were dissolved in TE buffer (90  $\mu$ l), and subjected to gel filtration using MicroSpin S-400 HR Column to remove the cleaved adapters. The eluted solution, which

contained the amplification products whose adapters were removed, was precipitated with ethanol, and the precipitates were dissolved in TE buffer at 1/2 concentration (10  $\mu$ l, 5 mM Tris-HCl, pH 7.5, 0.5 mM EDTA). Absorbance at 260 nm of this solution was measured to determine the yield. It was thought that tester specific genes were contained in this fraction.

#### (12) Re-subtraction

The candidate DNA fraction (1  $\mu$ g) of the tester specific genes obtained in the above procedure was added with T4 DNA ligase buffer at 10-fold concentration (3  $\mu$ l), adapters N-Bam24 (13.2  $\mu$ l), and N-Bam12 (6.6  $\mu$ l), and filled up to a total volume of 28.5  $\mu$ l, and then its temperature was lowered from 50°C to 10°C over one hour. Then, the mixture was added with T4 DNA ligase (1.5  $\mu$ l, 400 U/ $\mu$ l), and allowed to react at 16°C overnight to ligate the adapters. Then, the mixture was treated at 70°C for 10 minutes to inactivate the T4 DNA ligase, and after removing proteins in a conventional manner using a mixture of phenol and chloroform, subjected to precipitation with ethanol. The supernatant (17  $\mu$ l, about 0.5  $\mu$ g as DNA) was added with driver B1 DNA (40  $\mu$ g) and mixed, and then precipitated with ethanol. The precipitates were added with TE buffer at 3-fold concentration (4  $\mu$ l) and dissolved therein, overlaid with mineral oil (30  $\mu$ l, Sigma), and heat-treated at 98°C for one minute to denature the DNA. After the denaturation, the mixture was immediately cooled with ice, and added with 5 M sodium chloride (1  $\mu$ l, Wako Pure Chemicals Industries) to perform hybridization at 67°C for about 21 hours. From the sample after the hybridization, nucleic acids were precipitated with ethanol in the same manner as described above. The precipitates were added with TE buffer of 1/2 concentration (20  $\mu$ l) and dissolved therein, and to 2  $\mu$ l of this solution was added EX Taq DNA polymerase buffer at 10-fold concentration (20  $\mu$ l), 2.5 mM dNTP (240  $\mu$ l), water (147  $\mu$ l), and TaKaRa Ex Taq DNA polymerase (1  $\mu$ l), and allowed to react at 72°C for 5 minutes so that the nucleic acid should be blunt-ended. This sample was added with N-Bam24 (5.3  $\mu$ l, 10 OD/ml) as a primer, and PCR was performed with the condition that a cycle of 95°C for 3 minute and 70°C for 3 minutes was repeated for 10 cycles, and the mixture was left at 70°C for 7 minutes and then at 4°C. From the amplified sample, nucleic acids were extracted in the same manner as described above using a mixture of phenol and chloroform, added with glycogen, and precipitated with ethanol. This nucleic acid fraction was



added with TE buffer of 1/2 concentration (10  $\mu$ l), and dissolved therein. 1  $\mu$ l was taken from the solution, and added with TaKaRa Ex Taq DNA polymerase at 10-fold concentration (1  $\mu$ l), and PCR was performed with the condition that a cycle of 95°C for 1 minute and 70°C for 3 minutes was repeated for 20 cycles, followed by treatment at 70°C for 7 minutes and cooling to 4°C. By the above procedure, the tester specific genes were further screened. The adapters were removed again from the DNA fraction obtained from this procedure, and the same procedure was repeated once more utilizing J-Bam24 and J-Bam12 as adapters to afford final candidates of the tester specific gene, Clone #22.

#### (13) Isolation of Clone #22

The nucleotide sequences of the tester specific gene candidates obtained by the above procedure were determined as follows to isolate novel viral gene Clone #22. The #22 has *Sau3AI* cleaved sequences at the both ends. By utilizing these sequences, it was cloned into pT7BlueT vector (Navagen). This clone was transfected into *E. coli* TG-1, and transformant cells were screened. The plasmid DNA of the obtained transformants were analyzed. That is, for 60 clones in total, plasmid DNAs were prepared, and their nucleotide sequences were determined in the original direction and the reverse direction by using Thermo Sequencer Fluorescent-labelled primer cycle sequencing kit (Amersham International plc. Buckinghamshire, England). Based on these sequences, the clones were classified. As a result, 13 clones having the same nucleotide sequence were obtained. Consensus sequence was searched by aligning the sequences of these clones, and the sequence shown in SEQ ID NO: 11 was obtained. The clone having this sequence was designated as Clone #22. The full length of Clone #22 was 500-nucleotide length.

#### Example 2: HNT22 gene detection method (1)

A plurality of oligonucleotides of 20-nucleotide length constituting the nucleotide sequence of HNT22 Clone #22 obtained in Example 1 were produced, and various combinations thereof were examined for their utility as primers for gene amplification. That is, PCR was performed for samples which were positive for the genes and from which the genes were isolated in order to search sequences and combinations thereof capable of efficiently amplifying the genes. As a result, it was found that oligonucleotides having

the sequences shown in SEQ ID NO: 2 (primer name: RD037) and SEQ ID NO: 3 (primer name: RD038) as well as oligonucleotides having the sequences shown in SEQ ID NO: 4 (RD051) and SEQ ID NO: 5 (RD052) could be effectively used as primers for gene amplification. RD037 and RD051 are sense primers, and RD038 and RD052 are anti-sense primers. Amplification is performed by using a pair of sense primer and anti-sense primer.

The details of the method used for detecting genes are as follows.

Nucleic acids were extracted from blood serum or plasma (100  $\mu$ l) using a commercially available nucleic acid extraction kit (EX R&D, Sumitomo Metal Industries). Because it had been revealed that HNT22 virus is a DNA virus as described hereinafter (Example 8), viral genes were treated as DNA in this procedure. The extracted DNA was dissolved in TE buffer (10  $\mu$ l), and the whole volume was used as a sample. AmpliTaq DNA polymerase buffer at 10-fold concentration (5.0  $\mu$ l, Perkin Elmer), 10 mM dNTP (1.0  $\mu$ l), primers RD037 and RD038 (0.5  $\mu$ l for each, 10 OD/ml), and thermostable DNA polymerase (0.25  $\mu$ l, AmpliTaq DNA Polymerase, Perkin Elmer) were introduced into a tube exclusively for PCR, and added with distilled water to a total volume of 50  $\mu$ l. This reaction mixture was prepared upon use.

To the tube containing the reaction mixture, the above extracted DNA sample (10  $\mu$ l) was added, overlaid with mineral oil (50  $\mu$ l), stirred, and then centrifuged in a refrigerated centrifugal separator at 6000 rpm for 30 seconds. After the centrifugation, the tube was mounted on a thermal cycler, and PCR was performed. PCR was performed with a treatment at 95°C for 2 minutes and 30 seconds, then a cycle of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 45 seconds for 35 cycles, and a treatment at 72°C for 7 minutes after the last cycle.

When further amplification was performed, AmpliTaq DNA polymerase buffer at 10-fold concentration (5.0  $\mu$ l, Perkin Elmer), 10 mM dNTP (1.0  $\mu$ l), primers RD051 and RD052 (0.5  $\mu$ l for each, 10 OD/ml) that were selected from a nucleotide sequence located more interior than the aforementioned RD037 and RD038, and thermostable DNA polymerase (0.25  $\mu$ l, AmpliTaq DNA Polymerase, Perkin Elmer) were introduced into another tube exclusively for PCR, and further added with distilled water (37.5  $\mu$ l) to a total volume of about 45  $\mu$ l. To this mixture, the aforementioned amplification product (5  $\mu$ l) was added to afford a reaction mixture. This

reaction mixture was prepared upon use.

After the preparation of the reaction mixture, the mixture was overlaid with mineral oil (50  $\mu$ l), mixed, and then centrifuged in a refrigerated centrifugal separator at 6000 rpm for 30 seconds. Then, the tube was mounted on a thermal cycler, and PCR was performed. PCR was performed with a cycle of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds for 25 cycles, and a treatment at 72°C for 7 minutes after the last cycle.

The amplified genes were detected by electrophoresis. That is, 10  $\mu$ l of the amplification product obtained by the amplification with the above condition was taken, and subjected to agarose gel electrophoresis (2.5% NuSieve: Agarose EP = 3:1). The agarose gel after the electrophoresis was stained with ethidium bromide, and presence or absence of the amplification product was confirmed under ultraviolet light. The amplification product could be detected as a band at 270 bp when only the primers RD037 and RD038 were used, or as a band at 197 bp when the amplification was performed by using the primers RD051 and RD052 once or with additional amplification.

#### Example 3: HNT22 gene detection method (2)

While HNT22 viral infection cases in Japanese patients could be sufficiently detected by the HNT22 gene detection method of Example 2, in order to further examine conservation of the amplified region, sequences within the range containing the region of a large number of samples were amplified by the method using primers NG001/RD038 (1st PCR) and NG001/RD052 (2nd PCR) described hereinafter in Example 7, and examined. As a result, it was found that a plurality of genotypes including the two major Genotypes I and II (designations decided by the present inventors as described in Example 12 hereinafter) exist for HNT22 virus. It was further found that mismatches are scattered in the sequences of the primers RD037 and RD051, in particular at the 3' end side, in the genotype designated as Genotype II (Fig. 1 to Fig. 5). Based on an assumption that more specific and more sensitive gene detection methods can be developed by avoiding this mismatch sequence, further highly conserved sequences were searched. As a result, it was found that oligonucleotides having the sequences shown in SEQ ID NO: 6 (primer name: NG059), SEQ ID NO: 7 (NG061), and SEQ ID NO: 8 (NG063) can be utilized as primers for gene amplification (Fig. 1 to Fig.

5), and novel HNT22 gene detection methods utilizing them have been completed.

The details of the method used for detecting genes are as follows.

DNA was extracted from blood serum or plasma (50  $\mu$ l) in the same manner as in Example 2. The extracted DNA was dissolved in distilled water (20  $\mu$ l), treated at 95°C for 15 minutes, and immediately cooled in ice for 2 minutes to afford a sample for measurement. AmpliTaq DNA polymerase buffer at 10-fold concentration (5.0  $\mu$ l, Perkin Elmer), 2.5 mM dNTP (4  $\mu$ l), primers NG059 and NG063 (0.5  $\mu$ l for each, 10 OD/ml), and thermostable DNA polymerase (0.25  $\mu$ l, AmpliTaq DNA Polymerase, Perkin Elmer) were introduced into a tube exclusively for PCR, and further added with distilled water (29.75  $\mu$ l) to a total volume of 40  $\mu$ l. To the tube containing the reaction mixture, the extracted DNA sample (10  $\mu$ l) was added, overlaid with mineral oil (50  $\mu$ l), stirred, and then centrifuged in a refrigerated centrifugal separator at 6000 rpm for 30 seconds. After the centrifugation, the tube was mounted on a thermal cycler, and PCR was performed. PCR was performed with a cycle of 94°C for 30 seconds, 60°C for 45 seconds, and 72°C for 45 seconds for 35 cycles, and finished with a reaction at 72°C for 7 minutes after the last cycle.

When further amplification was performed, AmpliTaq DNA polymerase buffer at 10-fold concentration (5.0  $\mu$ l, Perkin Elmer), 2.5 mM dNTP (4  $\mu$ l), primers NG061 and NG063 (0.5  $\mu$ l for each, 10 OD/ml), and thermostable DNA polymerase (0.25  $\mu$ l, AmpliTaq DNA Polymerase, Perkin Elmer) were introduced into another tube exclusively for PCR, and further added with distilled water (37.75  $\mu$ l) to a total volume of 48  $\mu$ l. To this mixture, the aforementioned amplification sample (2  $\mu$ l) was added, and the mixture was overlaid with mineral oil (50  $\mu$ l), stirred, and then centrifuged in a refrigerated centrifugal separator at 6000 rpm for 30 seconds. Then, the tube was mounted on a thermal cycler, and PCR was performed. PCR was performed with a cycle of 94°C for 30 seconds, 60°C for 45 seconds, and 72°C for 45 seconds for 25 cycles, and finished with a reaction at 72°C for 7 minutes after the last cycle. The amplified genes were detected by agarose gel electrophoresis in the same manner as in Example 2. In this example, the HNT genes could be detected as a band at 286 bp when amplified by PCR using the first primers NG059 and NG063, or as a band at 271 bp when amplified by PCR using the primers NG061 and NG063.

Each PCR constituting the two-step PCR performed in this example

may of course be performed alone.

Further, because the gene detection method of Example 3 can be utilized for amplification of many different genes of the HNT22 gene including Genotypes I and II, and amplification specific for Genotype 1 can be realized by the gene amplification method of Example 2, it is possible to classify samples for their genotypes by subjecting each one sample to gene detection according to Example 2 and Example 3, and examining resulting detection patterns (Fig. 1 to Fig. 5).

#### Example 4: Extension of identified gene sequence

An identified gene sequence was extended based on Clone #22. That is, by using a sample (serum) of a blood donor exhibiting abnormality of hepatic function (34 years old, male, ALT [alanine aminotransferase] value: 106 IU), which sample showed high HNT22 virus titer (HNT22 genes could be detected even in  $10^{4-5}$ -fold dilution) as determined by the two-step PCR consisting of a combination of PCR utilizing the primer pair of RD037 and RD038 and PCR utilizing the primer pair of RD051 and RD052, and the two-step PCR consisting of a combination of PCR utilizing the primer pair of NG059 and NG063 and PCR utilizing the primer pair of NG061 and NG063, which were established in Examples 2 and 3, the #22 sequence was extended in the 5' direction and the 3' direction by the walking technique. The virus strain having the obtained nucleotide sequence (SEQ ID NO: 1) was designated as T278.

The details of the walking method performed in this example were as follows (Fig. 6).

##### (1) Sequencing of Clones T4 and T6

Two-step single-sided PCR was performed using sense and anti-sense primers specific for the nucleotide sequence of Clone #22 and non-specific primers which had nucleotide sequences of 41-nucleotide length shown in SEQ ID NOS: 12-15 (SSP-G, SSP-A, SSP-T and SSP-C).

For the extension of the 5' end, two-step single-sided PCR utilizing a combination of either of specific anti-sense primer RD038 or RD052 and one of the above-mentioned non-specific primers was performed. In the PCR of the first step (1st PCR), a cycle of 94°C for 30 seconds, 42°C for 45 seconds, and 72°C for 2 minutes was repeated 5 times, the reaction products were purified by SizeSep-400 Column (Pharmacia Biotechnology), and a cycle of 94°C for 30 seconds, 55°C for 45 seconds, and 72°C for 2 minutes was

repeated 35 times for amplification. In the PCR of the second step (2nd PCR), 1/10 volume of the product of the 1st PCR was used, and a cycle of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 2 minutes was repeated 30 times. PCR was performed by using TaKaRa Ex Taq DNA Polymerase (Takara Shuzo). The product was cloned into pT7Blue T vector in the same manner as in Example 1, and nucleotide sequences of the both strands were determined for three or more clones to obtain the sequence shown in SEQ ID NO: 16 (corresponding to nt1455-2257 shown in SEQ ID NO: 1, Clone T4), and the sequence shown in SEQ ID NO: 17 (corresponding to nt2061-3265 of SEQ ID NO: 1, Clone T6). The sequence shown in SEQ ID NO: 16 was obtained from amplification product of PCR utilizing the combination of the primers SSP-A and RD052, and the sequence shown in SEQ ID NO: 17 was obtained from amplification product of PCR utilizing the combination of the primers RD051 and SSP-C.

## (2) Sequencing of Clones T7, T9, T11, and T13

Using the newly identified Clones T6 and T4, the 5' nucleotide sequence was identified from Clone T4 and the 3' nucleotide sequence from Clone T6 in the same manner as in the above (1). That is, for the 5' sequence, two-step single-sided PCR was performed by using specific anti-sense primers which have the sequences of SEQ ID NOS: 18 and 19 having a sequence specific for the 5' end of Clone T4 (NG012 and NG013) and non-specific primers which have the nucleotide sequences of SEQ ID NOS: 12-15 and 20, resulting amplification products were cloned, and sequenced by the method described in Example 1. Specifically, PCR of the first step was performed by using a combination of the anti-sense primer NG012 and any one of the aforementioned non-specific primers. For PCR, a cycle of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 2 minutes was repeated 5 times, the reaction products were purified by SizeSep-400 Column (Pharmacia Biotechnology), and a reaction cycle of 94°C for 30 seconds, 55°C for 45 seconds, and 72°C for 2 minutes was further repeated 35 times. The PCR of the second step was performed by using 1/10 volume of the product of the PCR of the first step and NG013 as the specific anti-sense primer, and repeating a cycle of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 2 minutes 30 times. The amplification product was cloned into pT7Blue T vector in the same manner as in Example 1, and nucleotide sequences of the both strands were determined for three or more clones to obtain the sequence

shown in SEQ ID NO: 21 (corresponding to nt945-1575 in SEQ ID NO: 1, Clone T9) (Fig. 6).

For the 3' sequence, sequence amplification by utilizing two-step single-sided PCR similar to the above one using specific sense primers NG006 and NG010 which have the sequences of SEQ ID NOS: 22 and 23 having a sequence specific for the 3' end of Clone T6 and non-specific primers which have the nucleotide sequences of SEQ ID NOS: 12-15 and 20, cloning into pT7blue T vector, and sequence analyses were performed to afford the sequence shown in SEQ ID NO: 24 (corresponding to nt3126-3739 in SEQ ID NO: 1, Clone T7) (Fig. 6).

For the 5' sequence, the same procedure was further repeated to obtain the sequence shown in SEQ ID NO: 25 (corresponding to nt723-1024 of SEQ ID NO: 1, Clone T11) by using the sequence of the aforementioned Clone T9, and the sequence shown in SEQ ID NO: 26 (corresponding to nt1-831 shown in SEQ ID NO: 1, Clone T13) by using the sequence of Clone T11. By aligning the sequences of these clones, the sequence of HNT22 shown in SEQ ID NO: 1 was obtained. In order to obtain Clone T11, an oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 27 (primer name: NG031) was used as an as anti-sense primer for the single-sided PCR of the first step, and an oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 28 (primer name: NG032) was used as an as anti-sense primer for the single-sided PCR of the second step. In order to obtain Clone T13, an oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 29 (primer name: NG045) was used as an as anti-sense primer for the single-sided PCR of the first step, and an oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 30 (primer name: NG039) was used as an as anti-sense primer for the single-sided PCR of the second step. As non-specific primers, the nucleotide sequences of SEQ ID NOS: 12-15 and 20 were used as above (Fig. 6).

In order to establish the sequences, the sequences corresponding to nt1109-1575 (SEQ ID NO: 31, Clone T10), nt12-759 (SEQ ID NO: 32, Clone T14), and nt3119-3315 (SEQ ID NO: 33, Clone T8) were amplified by using oligonucleotides each having the nucleotide sequence shown in SEQ ID NO: 34 (primer name: NG013) and the nucleotide sequence shown in SEQ ID NO: 35 (primer name: NG025), oligonucleotides each having the nucleotide sequence shown in SEQ ID NO: 36 (primer name: NG040) and the nucleotide sequence shown in SEQ ID NO: 37 (primer name: NG046), and

oligonucleotides each having the nucleotide sequence shown in SEQ ID NO: 38 (primer name: RD057) and the nucleotide sequence shown in SEQ ID NO: 39 (primer name: RD058) as the primer pair, respectively, amplification products were cloned as above, and the sequences were analyzed (Fig. 6).

The sequences of the obtained clones were combined to afford the sequence of the full length 3739 bp shown in SEQ ID NO: 1. Upon constructing the sequence, when mutations were observed in sequences overlapped between clones (different among clones), preference was given to the most frequent nucleotide.

For the sequence of nt1455-3054 in the obtained sequence, known gene sequences were searched based on homology by using databases (DDBJ, National Genetics Institute, search programs: BLAST and FASTA). Among the 20 genes exhibiting the highest homology, only Simian cytomegalovirus major immediate early transcription unit IE94 was derived from viruses, and its homology was 50.7% even for the fragment of 383-nucleotide length which exhibited the highest homology.

From the above, it was confirmed that any viral genes exhibiting high homology to the gene sequences according to the present invention over the full length have not been known, and the genes found by according to the present invention have novel sequences.

**Example 5: Detection of HNT22 genes in blood donors who were negative for non-B, non-C, non-G hepatitis virus markers and exhibited abnormal alanine aminotransferase values and chronic hepatitis patients**

For 207 persons who were negative for known hepatitis virus markers and exhibited activity of alanine transferase (ALT) of 100 international unit (IU/l) or more, 26 persons who exhibited activity of  $\gamma$ -glutamyl transpeptidase ( $\gamma$ -GTP) of 500 IU/l or more, and 15 cases of non-B, non-C type chronic hepatitis all found in Japanese blood donors, presence of the HNT22 genes was examined by using the gene detection method of Example 2.

As controls, 88 blood donors with normal hepatic function and 22 patients of chronic hepatitis C were also examined.

As a result, the HNT22 genes were found in 16 persons out of the 207 persons with abnormally high ALT values (7.7%), 4 persons out of the 26 persons with abnormally high  $\gamma$ -GTP values (15.4%), and 3 patients out of the 15 non-B, non-C type chronic hepatitis patients (20%).



On the other hand, the HNT22 genes were found in 3 persons out of the 88 blood donors with normal hepatic function (3.4%), and 2 patients out of the 22 patients of chronic hepatitis C (9.1%).

**Example 6: Detection of HNT22 genes in post-transfusion hepatitis cases**

For post-transfusion hepatitis cases negative for known hepatitis virus markers, presence and timing of appearance of the HNT22 genes were examined in blood of patients before blood transfusion, after blood transfusion and before crisis of hepatitis, after crisis of hepatitis, and blood for transfusion (if possible) by the method of Example 2.

**(1) Case 1**

Post-transfusion hepatitis case, male, 63 years old.

This case was negative for any of markers of HBV, HCV and GBV-C/HGV throughout the examination period. ALT value, which is a hepatic function marker, was normal before blood transfusion, but abnormal values were observed eight weeks and nine weeks after the blood transfusion. Blood of this case was assayed for the HNT22 genes before the blood transfusion (represented as 0 week after blood transfusion) and 6, 8, 9, 10, 11, 12, 15, and 24 weeks after the blood transfusion. As a result, the HNT22 genes were detected from patient blood serum even before the blood transfusion (Table 1). The blood transfused to this case consisted of 4 units. When presence of the HNT22 genes in these transfusion blood units was examined, the HNT22 genes were detected in 1 unit.

Table 1

Blood collection (week)	ALT (IU/L)	HCV antibody	GBV-C/HGV RNA	HNT22 gene	
				1st PCR	2nd PCR
0	11	-	-	+	+
6	62	-	-	NT	NT
8	109	-	-	NT	NT
9	443	-	-	+	+
10	148	-	-	+	+
11	41	-	-	-	+
12	20	-	-	-	+
15	33	-	-	+	+
24	19	-	-	-	+

NT: not tested

## (2) Case 2

Post-transfusion hepatitis case, male, 58 years old.

This case was negative for any of markers of HBV, HCV and GBV-C/HGV throughout the examination period. ALT value, which is a hepatic function marker, was normal before blood transfusion, but abnormal values were observed around 10 weeks after the blood transfusion. Blood of the patient of this case was assayed for the HNT22 genes 2, 6, 8, and 10 weeks after the blood transfusion. As a result, the HNT22 genes were detected from patient blood sera at 6, 8 and 10 weeks after the blood transfusion in the 1st PCR of the first amplification step (Table 2). In contrast, at 2 weeks, the titer was low, and detected only in the 2nd PCR. The blood transfused in this case was not preserved, and therefore presence of the HNT22 genes in the transfused blood could not be confirmed.

This example is a case where Clone #22 of HNT22 was isolated. It was found that the virus was present in the blood at two weeks which was used as a driver for the isolation. However, because it could be detected only in the highly sensitive second step of the amplification, the amount of the virus at that point was quite small, and therefore it substantially functioned as a driver.

Table 2

Blood collection (week)	ALT (IU/L)	HCV antibody	GBV-C/HGV RNA	HNT22 gene	
				1st PCR	2nd PCR
2	36	-	-	-	+
6	37	-	-	+	+
8	55	-	-	++	+
10	180	-	-	++	+

## (3) Case 3

Post-transfusion hepatitis case, male, 56 years old.

This case was also negative for any of markers of HBV, HCV and GBV-C/HGV throughout the examination period. ALT value, which is a hepatic function marker, was normal before blood transfusion, but abnormal values were observed around 6 weeks after the blood transfusion. Blood of the patient of this case was assayed for presence of the HNT22 genes before the blood transfusion (represented as 0 week after blood transfusion) and 2, 4, 6, 8, 12, 13 weeks, 4 and 5 months after the blood transfusion by utilizing PCR comprising the two-step amplification according to the method of Example 2. Serial 10-fold dilutions of the samples were also prepared and assayed in the same manner, thereby the titer of the viral genes (considered to be the amount of the virus) was determined from the maximum dilution ratios where the gene was detected. As a result, while the blood 2 weeks after the blood transfusion was negative, the HNT22 genes were detected in each blood 6 weeks to 4 months after the blood transfusion (Table 3). On the other, aberration of hepatic function became significant from 6 weeks after the blood transfusion, and then gradually ameliorated, and this process was well conformed to the variation of the viral amount. The blood transfused in this case was not preserved, and therefore presence of the HNT22 genes in the transfused blood could not be confirmed.

Table 3

Blood collection (week)	ALT (IU/L)	HCV antibody	GBV-C/HGV RNA	HNT22 gene	
				1st PCR	2nd PCR
0	17	-	-	-	-
2	36	-	-	-	-
4	34	-	-	-	+
6	192	-	-	-	+
8	172	-	-	-	+

## (4) Case 4

Post-transfusion hepatitis case, female, 70 years old

This case was also negative for any of markers of HBV, HCV and GBV-C/HGV throughout the examination period. ALT value, which is a hepatic function marker, was normal before blood transfusion, but abnormal values was observed around 6 weeks after the blood transfusion. Blood of the patient of this case was assayed for presence of the HNT22 genes 4, 6, 8, 11, 12, 15, 16, 17 and 21 weeks after the blood transfusion by utilizing PCR comprising the two-step amplification according to the method of Examples 2 and 3, and presence of the HNT22 genes and the titer of the viral genes were also determined as in Case 3 (similar results were obtained by the methods of Examples 2 and 3). As a result, while the blood at 4 weeks after the blood transfusion was negative, the titer increased from 6 weeks after the blood transfusion, and the highest titer was observed at 11 weeks when the aberration of hepatic function was the most significant. Then, as the virus disappeared, the hepatic function was normalized, and thus the variations of the viral amount and the hepatic function were well conformed to each other (Table 4). The blood transfused in this case was not preserved, and therefore presence in the HNT22 genes in the transfused blood could not be confirmed. When presence of HNT antibodies was assayed by the method described in Example 13, it was positive after the crisis.

Table 4

Blood collection (week)	ALT (IU/L)	HCV antibody	GBV-C/HGV RNA	HNT22 gene		HNT antibody
				1st PCR	2 <sup>nd</sup> PCR	
4	14	-	-	-	-	-
6	15	-	-	-	+	NT
8	10	-	-	+	+	NT
11	140	-	-	++	+	NT
12	36	-	-	-	+	NT
15	16	-	-	-	-	NT
16	7	-	-	-	-	NT
17	4	-	-	-	-	NT
21	7	-	-	-	-	+

NT: not tested

#### Example 7: Analysis of blood transfusion infection cases

The facts that the HNT22 genes of the present invention were detected in Example 6 in the cases of post-transfusion hepatitis which was negative for known hepatitis markers and whose cause was indistinct, and that the variation of abnormal value of the hepatic function marker well conformed to the variation of the viral amount strongly suggested that HNT22 was a cause of the hepatitis. This example was performed aiming at more clearly demonstrating that HNT22 is transmitted by blood transfusion. That is, for cases having blood transfusion history where the HNT22 genes were detected in blood after blood transfusion and all of the blood pilots used for the transfusion were preserved, blood after blood transfusion and the blood pilots were assayed for the genes by the method described hereinafter to identify blood transfusion pilots positive for the gene and considered to be the source of infection. Further, sequences of the genes obtained from the patients' blood and the pilots were determined, and compared to examine their sequence homology.

For the cases determined positive for the HNT22 genes by the method described in Example 2 after blood transfusion, it was determined if the HNT22 genes could be detected in blood before blood transfusion and blood pilots used for blood transfusion. As a result, while the HNT22 genes could not be detected in blood samples before blood transfusion, it was detected in all samples at 2 weeks after blood transfusion and thereafter.

Therefore, it was strongly suggested that the infection was brought by the blood transfusion.

Further, all of the ten blood pilots used were assayed for the HNT22 genes, and the HNT22 genes were detected in one pilot among them. Nucleic acids were extracted from all of the samples positive for HNT22 according to the method described in Example 2. Then, they were amplified as in Example 2 by replacing the sense primer RD037 in the 1st PCR of Example 2 with the primer NG001 having the nucleotide sequence shown in SEQ ID NO: 40, and changing the PCR condition to 35 cycles of a cycle of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute. When amplified under the above conditions, a DNA fragment of 415 bp was provided in the positive cases. The second step of the amplification in Example 2 was performed by replacing the sense primer RD051 with NG001, and using PCR condition of a reaction cycle of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute repeated 25 times. When amplified under these conditions, a DNA fragment of 396 bp was provided in the positive cases. This 396 bp amplification product was cloned by inserting it into a plasmid vector as in Example 1, and nucleotide sequences were determined and compared for 3 clones for each amplification to examine homology.

As a result, the sequences of the clones obtained from the blood pilots for blood transfusion that were positive for HNT22 were completely conformed with the HNT22 gene sequence obtained from the patients transfused with the pilots (Fig. 7). This demonstrated that HNT22 virus was transmitted by blood transfusion, and became persistent. When this gene sequence was compared with the sequence of Clone #22, 3 nucleotides were different among 356 nucleotides other than those derived from the primers for the both ends.

**Example 8: Verification of the fact that the virus of the present invention is DNA virus**

As hepatitis viruses, there are DNA viruses like HBV, and RNA viruses like HCV. Which kind of viruses the virus of the present invention belongs to was determined by the following procedure.

(1) PCR which omits reverse transcription reaction step

As described in Example 1, the samples of the isolated genes of the present invention undergone a step of reverse transcription reaction after

the nucleic acid extraction. Accordingly, the samples after the reverse transcription reaction contains DNA which were originally present and DNA which had been present as RNA before the reaction and were converted into DNA by the reverse transcription reaction. Therefore, if the virus of the present invention is an RNA virus, detection of the gene of the virus should become impossible or quite difficult when the reverse transcription reaction is omitted.

Based on the above, the present inventors performed PCR according to the procedure of Example 2 that omitted the reverse transcription reaction to examine the above possibility. As a result, it was confirmed that the HNT22 genes could be detected at a similar level even without the reverse transcription reaction.

HNT22 was confirmed to be a DNA virus from the above.

## (2) Verification by deoxyribonuclease treatment

In addition to the analysis of the above (1), the present inventors treated a nucleic acid fraction extracted from a sample confirmed by the method of Example 2 to contain the HNT22 genes with a deoxyribonuclease, and subjected to reverse transcription reaction, and the resulted product was assayed as a sample for the HNT22 genes by the method of Example 2.

Specifically, a nucleic acid fraction extracted by using the same commercially available kit as Example 2 (EX R&D, Sumitomo Metal) was treated with DNase I (Takara Shuzo) at 37°C for 30 minutes, and then activity of DNase I was inhibited. For this sample, detection of the HNT22 genes was attempted by carrying out the procedure of Example 1 after the reverse transcription under the same condition. As a result, it was found that the HNT22 genes became undetectable after the treatment of DNase I.

The results of the above (1) and (2) demonstrated that HNT22 virus of the present invention is a DNA virus.

## Example 9: Verification of the fact that the virus of the present invention is a single-stranded DNA virus

### (1) Verification utilizing restriction endonuclease

It has been known that there are two kinds of viruses having different gene structures among DNA viruses, i.e., those having a single-stranded DNA and those having a double-stranded DNA. The present inventors considered it is important to determine which kind of the viruses

the virus of the present invention belongs to in view of elucidating the identity of the HNT22 virus, and performed the following experiment.

If it is assumed that the HNT22 virus gene exists in a double-stranded state in nature, there would be several sites specific for restriction endonucleases in its sequence. However, if the viral gene is single stranded, there would be no such restriction endonuclease specific sequences. Conversely, if extracted genes are susceptible to (cleaved by) restriction endonucleases specific for the above sequences, the genes of the virus of the present invention have a double-stranded structure.

Based on the above idea, the present inventors examined if there is a cleavage with a restriction endonuclease *EcoRI* for which a specific sequence would be present in the sequence of the HNT22 genes when they are assumed to be double-stranded, or with a restriction endonuclease *BglII* for which a specific sequence would not be recognized as control.

DNA (90  $\mu$ l) was obtained using the method described in Example 1 from plasma (800  $\mu$ l) obtained from patients confirmed to be positive for HNT22 virus by the methods of Examples 2 and 3. A 15  $\mu$ l aliquot of each DNA was taken, added with 2  $\mu$ l of *EcoRI* (Takara Shuzo) or *BglII* (Takara Shuzo), and incubated at 37°C for 2.5 hours. In parallel, the same reaction was performed by adding phosphate buffer instead of the restriction endonucleases. After the treatment with the restriction endonucleases, 3  $\mu$ l aliquot was taken from each sample, 10-fold and 100-fold dilution samples were prepared from it, and it was attempted to amplify a HNT22 gene region containing the *EcoRI* restriction endonuclease specific sequence for each sample by using the primers NG001 and RD038 which contained an *EcoRI* restriction endonuclease specific sequence within them. As a result, no cleavage by restriction endonuclease was recognized in any of the samples.

## (2) Verification by mung bean nuclease treatment

Mung bean nuclease has been known to specifically cleave a single-stranded DNA. Therefore, it is considered that if HNT22 exists as a single strand, it would be susceptible to mung bean nuclease.

Based on the above idea, the present inventors obtained DNA from the same patient used for the restriction endonuclease treatment experiment in the same manner, and treated with mung bean nuclease. As a control, a partial region of HNT22 amplified by the method of Example 2 was introduced into phage M13, and a double-stranded one and a single-stranded



one were prepared, and subjected to mung bean nuclease treatment. Then, the HNT22 genes were amplified according to the method of Example 2. As a result, the DNA obtained from the HNT22 positive patient and the single-stranded DNA derived from the phage could not be amplified, and thus they were susceptible to mung bean nuclease.

From the above results of (1) and (2), it was confirmed that the HNT22 genes exist as a single-stranded DNA, and HNT22 virus is a single-stranded DNA virus.

#### Example 10: Amino acid sequences encoded by HNT22 genes

Based on the HNT22 gene sequences identified in Example 4, the amino acid sequences encoded by the HNT22 genes were analyzed. Initiation codon sequence and termination codon sequence were searched in the nucleotide sequences obtained, and opening reading frame (ORF) of the HNT22 genes was searched by considering which combination thereof would afford an ORF in a usually expected size as a determination criterion. As a result, it was found that there were two ORFs, ORF1: nt589-nt2898 (770 amino acid residues), and ORF2: nt107-nt712 (202 amino acid residues) (Fig. 6 and Fig. 8). From the above, it can be assumed that the HNT22 genes encode polypeptides comprising the amino acid sequences shown in SEQ ID NOS: 9 and 10.

Based on these amino acid sequences, hydrophobicity/hydrophilicity characteristics of the encoded polypeptides were analyzed in a conventional manner (Fig. 9).

#### Example 11 Isolation of virus particles

Blood that had been found to be positive for the HNT22 genes by the method of Example 2 was fractionated by using sucrose density gradient centrifugation to analyze the density distribution of the HNT22 genes, and the possibility to be a virus particle was examined based on the density distribution analysis.

(1) Blood positive for the HNT22 genes (250  $\mu$ l) and plasma positive for HBV (5  $\mu$ l) as a control were mixed, and centrifuged at 15000 revolutions/minute for 5 minutes by using a refrigerated microcentrifugal separator to precipitate impurities, which were then removed.

(2) The supernatant obtained in the procedure of the above (1) (0.2 ml) was added to a density gradient carrier formed in a centrifugation tube for

Beckman SW60 rotor by overlaying layers of sucrose solution having gradient concentrations in the order of 60% (0.7 ml), 50%, 40%, 30%, 20% and 10% (0.2 ml for each) by weight from the bottom, and TEN buffer (2.3 ml, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5 M NaCl) was overlaid thereon.

(3) After centrifugation at 40000 revolutions/minute at 10°C for 45 hours, 300 µl portion was collected from the bottom of each tube, and subjected to density measurement using a refractometer.

(4) A 100 µl portion was collected from each density fraction, mixed with an extraction reagent (300 µl, Okamoto H. et al., J. Virol. 64:1298-1303, 1990) containing proteinase K (Boehringer Mannheim) and sodium dodecylsulfate (SDS, Wako Pure Chemicals Industries), and allowed to react at 70°C for 3 hours. The nucleic acid fraction was extracted by adding phenol, and further extracted with phenol and chloroform. Finally, the nucleic acids were precipitated by adding ethanol, and collected, and then dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, 1.0 mM EDTA) to a suitable concentration.

(5) By using the obtained DNA as samples, the HNT22 genes were amplified by the method of Example 2, and the HBV gene was amplified and detected as follows.

A DNA sample (5 µl) was added to a PCR tube containing an amplification reaction mixture comprising a primer S1-2 having the nucleotide sequence shown in SEQ ID NO: 41 and a primer S2-1 having the nucleotide sequence shown in SEQ ID NO: 42 (0.5 µl for each, 10 OD/ml), which were selected from sequences within a surface antigen gene region of HBV, 2.5 mM dNTP (4 µl), buffer for thermostable DNA polymerase at 10-fold concentration (5.0 µl, buffer attached to AmpliTaq DNA polymerase, Perkin Elmer), thermostable DNA polymerase (0.25 µl, AmpliTaq DNA Polymerase, Perkin Elmer), and distilled water (34.75 µl), overlaid with mineral oil (50 µl), stirred, and centrifuged at 5000 rpm for 30 seconds, and the tube was mounted on a thermal cycler to performed PCR. PCR was performed with a cycle of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 75 seconds repeated for 35 cycles. In this method, the HBV gene could be detected as a band of a length of 250 bp in electrophoresis gel. When more sensitive detection was required, second PCR was performed by using a portion of the above amplification product as follows. That is, the amplification product (5 µl) was added to the same reaction solution as above except that the primers used were changed to a primer \$088 having the

nucleotide sequence shown in SEQ ID NO: 43 and a primer S2-2 having the nucleotide sequence shown in SEQ ID NO: 44, and treated in the same manner as above. Then, PCR was performed with a cycle of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 60 seconds, which was repeated 25 times. The amplification product provided by the second PCR was detected in the electrophoresis gel as a band of 228 bp.

As a result of the above assay, the HNT22 genes existed as a peak at a sucrose concentration of 54.5%, and density of 1.26 g/cm<sup>3</sup>, whereas the HBV as the control existed as a peak at a density of 1.26-1.20 g/cm<sup>3</sup> as previously reported (Table 5). This density analysis indicated that the HNT22 genes existed in the virus particle fraction, and it was demonstrated that they can be collected in the density fraction mentioned above by density gradient centrifugation.

Table 5: Distribution of HNT22 genes in sucrose density gradient centrifugation fractions

Sucrose density (%)	Density (g/ml)	HNT22-DNA		HBV-DNA	
		1st PCR	2nd PCR	1st PCR	2nd PCR
60.7	1.29	+	+	-	+
54.5	1.26	++	+	+++	+
45.2	1.20	±	+	+++	+
30.9	1.13	±	+	++	+
19.5	1.08	-	+	+	+
12.0	1.05	-	-	+	+

-: Negative, ±: Weakly positive, +: Positive, ++: Fairly positive, +++: Strongly positive

#### Example 12: HNT22 genotype sequences

Because it had been suggested that HNT22 had several genotypes as mentioned in Example 3, the present inventors searched genotypes of HNT22 by analyzing the amplified genes of 22 HNT22 positive cases which had been determined positive by the gene detection method of Example 3, and preparing a molecular phylogenetic tree based on the gene sequences.

The present inventors examined Japanese, Americans, and Frenchmen for the presence of the HNT22 genes by the method described in Example 3, and as a result obtained 199 positive cases (187 Japanese cases,

8 American cases, 4 Frenchman cases). Amplified genes of these positive cases were cloned according to the method described in Example 1, and sequenced for at least 3 clones for each, and a phylogenetic tree was prepared by using a commercially available software (Fig. 10, sequences are shown in Figs. 11-12). As a result, the HNT22 genes were classified into 10 types having the sequences shown in SEQ ID NOS: 45-54 (corresponding to nt1939-2160 of SEQ ID NO: 1). The present inventors designated them as Genotypes 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10, respectively.

**Example 13: Anti-HNT22 antibody detection method by immunoprecipitation utilizing isolated HNT22 virus particles**

It was demonstrated that the HNT22 particles can be isolated and collected by the method of Example 11. The present inventors studied detection of antibodies by immunoprecipitation based on the above finding.

First, feces were collected from HNT infected patients who had been found to have the HNT22 genes in their blood, and suspended in a suitable solvent to prepare samples, and presence of the HNT22 genes in the feces was examined according to the method of Example 3. As a result, it was found that the HNT22 genes were also present in the feces, their buoyant density was 1.35 g/cm<sup>3</sup>, and the gene sequence was identical to the gene sequence of HNT22 obtained from blood of the same patients. Therefore, it was decided to collect HNT22 particles from feces.

Distilled water was added to feces obtained from a patient to prepare a 15% (weight concentration) suspension. This suspension was centrifuged at 3000 rpm for 30 minutes by using a refrigerated centrifugal separator (Hitachi) to afford a supernatant. This supernatant was further centrifuged at 10000 rpm for 5 minutes by using a refrigerated microcentrifugal separator (High Apeed Micro Refrigerated Centrifuge, Tomy Seikou). The supernatant was collected as HNT22 particle suspension. DNA titer of the particle suspension was 10<sup>5</sup>/ml.

The above HNT22 particle suspension (10 µl) was added to serum or plasma (50 µl), and allowed to react at 37°C for 24 hours. After the reaction, goat anti-human IgG (50 µl, #46340, Cappel) was added as second antibodies as its stock solution, and allowed to react at 37°C for one hour. After completion of the reaction, the mixture was centrifuged at 10000 rpm for 5 minutes using a refrigerated microcentrifugal separator (High Apeed Micro Refrigerated Centrifuge, Tomy Seikou) to be isolated into a supernatant and

precipitates. The supernatant was transferred into a separate Eppendorf tube, and DNA was extracted by using a commercially available nucleic acid extraction kit (EX R&D, Sumitomo Metal). On the other hand, physiological saline (110  $\mu$ l) was carefully added to the precipitates, and centrifuged at 10000 rpm for 5 minutes. The supernatant was discarded, and the residue was suspended in physiological saline (110  $\mu$ l) added dropwise. DNA was extracted from this suspension in the same manner as above. The obtained DNA fraction was dissolved in distilled water (20  $\mu$ l), and treated at 95°C for 5 minutes, and a half amount of the obtained solution was used as a sample for HNT22 gene detection. Detection of the HNT22 genes was performed in the same manner as in Example 3. The same serum or plasma treated in the same manner without adding the HNT22 particles, and serum or plasma negative for HNT22 treated in the same manner as above were used as controls.

As a result, the HNT22 genes were found in the precipitate fraction of the HNT22 gene positive blood serum or plasma, whereas the HNT22 genes were not found in the precipitate fraction when the particle suspension was not added, or HNT negative serum or plasma was used. From these results, it is considered that only when HNT22 antibodies are present, HNT22 particles aggregate with these antibodies and the anti-human IgG antibodies to form a precipitate fraction, and conversely, in a case where the HNT genes are present in the precipitate fraction, the anti-HNT antibodies are present.

**Example 14: Measurement of anti-HNT22 antibody in post-transfusion hepatitis cases and fulminant hepatic failure cases by anti-HNT22 antibody detection method utilizing HNT particle suspension**

The present inventors examined presence or absence of the anti-HNT22 antibodies for Case 4 mentioned in Example 6 who had transiently infected by HNT22 after blood transfusion and exhibited hepatic function aberration, and convalescence cases of sporadic fulminant non-A-G hepatic failure whose infection of known hepatitis viruses had been denied by using the method of Example 13 (Tables 4 and 6).

As a result, it was demonstrated that anti-HNT22 antibodies appeared after disappearance of the virus for the both cases, and exhibited appearance and disappearance pattern similar to that of the neutralizing antibodies observed in viral infection.

**Table 4: Appearance and disappearance of HNT22 gene and anti-HNT22 antibody in convalescence patients of sporadic fulminant non-A-G hepatic failure**

Blood collection (day)	ALT (IU/L)	HCV antibody	GBV-C/HGV RNA	HNT22 gene		HNT antibody
				1st PCR	2nd PCR	
0	2568	-	-	+	-	-
2	523	-	-	+	+	NT
7	152	-	-	++	+	NT
18	28	-	-	+	+	NT
31	16	-	-	-	+	NT
38	9	-	-	-	-	+

NT: not tested

After admission	ALT	HCV	GBV-C/HGV
Blood collection day (IU/l)	antibody	RNA	

#### **Example 15: Detection and sequencing of HNT22 gene subspecies**

Because it was demonstrated that a plurality of genotypes existed for HNT22 virus (Example 12), and other viruses such as hepatitis B and C viruses show regional bias of genotypes, major HNT22 genotypes in the U.S.A. may be different from the Japanese major types. Therefore, HNT22 positive samples obtained from Americans were examined.

Nucleic acids were extracted from 100 µl each of sera from Americans confirmed to be HNT22 virus positive by the method described in Example 3 (designated as UM3-17, UM3-34, UM3-56, and UM3-73, respectively) by using a commercially available nucleic acid extraction kit (High Pure Viral Nucleic Acid Kit, Boehringer Mannheim) according to the attached instructions to prepare samples.

PCR was performed by using an oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 55 (NG055) and an oligonucleotide having the nucleotide sequence shown in SEQ ID NO: 8 (NG063) selected based on the identified sequence of HNT22 virus as primers, and the samples obtained above as templates, and according to the following conditions. The locational relation of the primers used is shown in Fig. 13.

## PCR conditions

## (1) Reaction mixture composition

Template nucleic acid solution	10 $\mu$ l
10 $\times$ Ex. Taq buffer	5 $\mu$ l
2.5 mM dNTP	4 $\mu$ l
Primer (NG055: 10 OD/ml)	1 $\mu$ l (OD at 280nm, same shall apply hereinafter)
Primer (NG063: 10 OD/ml)	1 $\mu$ l
Distilled water	29.5 $\mu$ l
Ex. Taq DNA polymerase	1 $\mu$ l
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Total	50 $\mu$ l

## (2) Reaction condition

95°C	2 minutes	} 35 cycles
95°C	45 seconds	
55°C	30 seconds	
72°C	90 seconds	
72°C	7 minutes	

As a result, gene amplification was obtained for all of the samples. A plurality of amplification fragments were cloned into T vector for each sample in the same manner as in Example 1, and the gene sequences were determined. As a result, from UM3-17 and UM3-73, clones exhibiting high homology to the already identified nucleotide sequences and, in addition, clones having a sequence exhibiting low homology to those sequences were obtained (referred to as U17-2 and U73-2 respectively hereinafter). Both of the sequences exhibited 30% homology to a corresponding region of the nucleotide sequences identified so far. From UM3-56 and UM3-34, only nucleotide sequences exhibiting high homology to the nucleotide sequences already identified were obtained.

An oligonucleotide (NT01) having the nucleotide sequence shown in SEQ ID NO: 56 was prepared as a primer for further amplifying a nucleotide sequence of 5' end of the above clone, based on comparison of sequences between the U17-2 clone obtained above and HNT22 virus identified so far, and selection of a highly conserved region. PCR was performed by using this oligonucleotide NT01 and an oligonucleotide having the nucleotide

sequence shown in SEQ ID NO: 57 (NG054) prepared based on the sequence of HNT22 virus already identified as primers, and a nucleic acid derived from UM3-17 as a template, and according to the following conditions. The obtained amplification fragment was sequenced in the same manner as described above. The locational relation of the primers used is shown in Fig. 13.

#### PCR conditions

##### (1) Reaction mixture composition

Template nucleic acid solution	10 $\mu$ l
10 $\times$ Ex. Taq buffer	5 $\mu$ l
2.5 mM dNTP	4 $\mu$ l
Primer (NT01: 10 OD/ml)	1 $\mu$ l
Primer (NG054: 10 OD/ml)	1 $\mu$ l
Distilled water	29.5 $\mu$ l
Ex. Taq DNA polymerase	1 $\mu$ l
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Total	50 $\mu$ l

##### (2) Reaction condition

95°C	2 minutes	} 35 cycles
95°C	45 seconds	
55°C	30 seconds	
72°C	90 seconds	
72°C	7 minutes	

On the other hand, oligonucleotides having the nucleotide sequences shown in SEQ ID NOS: 58 and 59 (primers NT03 and NT04, respectively) were prepared as primers for further amplifying a sequence of 3' end of the above clones, based on the 3' end nucleotide sequence of U17-2 and the already identified sequence of HNT22 virus, and oligonucleotides having the nucleotide sequences shown in SEQ ID NOS: 60 and 61 (primer NG065 and NG021, respectively) were prepared as primers for amplification, based on an already identified sequence around the 3' end of the nucleotide sequence of HNT22 virus. First, gene of 3' end was amplified by using NT03 and NG065, and a part of the obtained amplification fragment was amplified by using NT04 and NG065 or NG021. The locational relation of the primers



used is shown in Fig. 13. The amplification was performed with the following conditions by using a nucleic acid derived from UM3-17 as a template. The nucleotide sequence of the amplification fragment was determined in the same manner as described above.

### PCR conditions

#### First step PCR

##### (1) Reaction mixture composition

Template nucleic acid solution	10 $\mu$ l
10 $\times$ Ex. Taq buffer	5 $\mu$ l
2.5 mM dNTP	4 $\mu$ l
Primer (NG03: 10 OD/ml)	1 $\mu$ l
Primer (NG065: 10 OD/ml)	1 $\mu$ l
Distilled water	29.5 $\mu$ l
Ex. Taq DNA polymerase	1 $\mu$ l
<hr/>	
Total	50 $\mu$ l

##### (2) Reaction condition

95°C	2 minutes	} 35 cycles
95°C	45 seconds	
55°C	30 seconds	
72°C	90 seconds	
72°C	7 minutes	

#### Second step PCR

##### (1) Reaction mixture composition

Template nucleic acid solution	5 $\mu$ l
10 $\times$ Ex. Taq buffer	5 $\mu$ l
2.5 mM dNTP	4 $\mu$ l
Primer (NT04: 10 OD/ml)	0.5 $\mu$ l
Primer (NG065: 10 OD/ml) or Primer (NG021: 10 OD/ml)	0.5 $\mu$ l
Distilled water	34.5 $\mu$ l
Ex. Taq DNA polymerase	0.5 $\mu$ l
<hr/>	
Total	50 $\mu$ l

## (2) Reaction condition

95°C	2 minutes	} 20 cycles
95°C	45 seconds	
55°C	30 seconds	
72°C	90 seconds	
72°C	7 minutes	

From the above procedure, clones 1-1, 1-9 and 1-10 were obtained as 5' end sequence clones of U17-2, and 2A-3 (when the primers in the second step PCR were the combination of NT04/NG065), and 2B-1 and 2B-3 (when the primers in the second step PCR were the combination of NT04/NG021) were obtained as 3' end sequence clones. Nucleotide sequences of these clones were determined in the same manner as described above, and ligated to obtain a nucleotide sequence of virus gene detected from sera derived from Americans (SEQ ID NO: 62).

By comparing this sequence with the nucleotide sequences of the HNT22 genes, it was found that extremely high homology can be observed for the 5' end and 3' end sequences whereas homology of the other sequences was as low as around 30%, and this sequence could not be detected by the detection methods of Examples 2 and 3. The virus having this gene was designated as TUS01.

The nucleotide sequence of the TUS01 gene was searched for ORF in the same manner as in Example 10. As a result, it was found that two ORFs, ORF1: nt590-nt2872 (761 amino acid residues) and ORF2: nt258-nt725 (156 amino acid residues) were present (Fig. 15). These existed at locations corresponding to those of ORF1 and ORF2 in the nucleotide sequence of the HNT22 genes.

Homology of each region was summarized in Table 7.

Table 7: Homology of HNT22 gene and TUS01 gene

	HNT22 <sup>2)</sup>	TUS01	Sequence homology
Full length	3739nt	3722nt	63.7%
5' untranslated region	262nt	257nt	90.3%
Translated region	2636nt	2615nt	54.7%
ORF1	2310nt	2283nt	54.8%
(Amino acid sequence)	770aa	761aa	37.0%
ORF2 <sup>1)</sup>	450nt	468nt	55.5%
(Amino acid sequence)	150aa	156aa	38.8%
3' untranslated region	841nt	850nt	84.2%

1) For ORF2, to make comparison based on TUS01, the frame from the second ATG codon was employed for HNT22.

2) Sequence used as HNT22 was that of TA278.

The results of the comparison of nucleotide sequences of highly homologous 5' untranslated region (5' end region) and 3' untranslated region (3' end region) are shown in Figs. 15 and 16.

When homology of the nucleotide sequence of the TUS01 gene with known sequences was determined by using FAST and BLAST in the same manner as in Example 4, all of those exhibiting high homology were nucleotide sequences of the HNT22 genes.

Judging from the above-mentioned characteristics, TUS01 virus is assumed to be a subspecies of HNT22 virus.

#### Example 16: Simultaneous detection of HNT22 gene and TUS01 gene

A method capable of simultaneously detecting the TUS01 gene, which cannot be detected by the methods of Examples 2 and 3, was studied.

By comparing the both sequences, as oligonucleotides containing a common sequence and considered to be suitable as PCR primers, NG054 and NG065 were selected. Nucleic acids were extracted from blood serum (100  $\mu$ l) according to the method described in Example 2. The extracted nucleic acids were dissolved in distilled water (10  $\mu$ l). The dissolved nucleic acids (10  $\mu$ l) were added to a tube exclusively for PCR containing solutions of NG054 and NG065 (1  $\mu$ l, 10 OD/ml for each), Ex. Taq buffer at 10-fold

concentration (5  $\mu$ l, TaKaRa Shuzo), 2.5 mM dNTP (4  $\mu$ l), and distilled water (29.5  $\mu$ l), immediately added with thermostable DNA polymerase (Ex. Taq Polymerase: TaKaRa Shuzo), and amplified by treatment at 95°C for 2 minutes, followed by a cycle of 95°C for 45 seconds, 55°C for 30 seconds, and 72°C for 120 seconds, which was repeated 35 times, and finally a reaction at 72°C for 7 minutes. The amplification products were separated by agarose gel electrophoresis, and presence of bands of lengths predicted from the nucleotide sequences was confirmed. The amplification products for which bands were confirmed were sequenced. As a result, it was found that the HNT22 gene and the TUS01 gene could be simultaneously detected by this method.

#### Industrial Applicability

According to the present invention, a so far unknown etiologic virus of blood-borne infectious hepatitis whose cause had been indistinct was identified. This enables establishing methods for gene assay, antigen assay, and antibody assay, and providing kits for test of the hepatitis, and vaccines for prevention thereof.